MOLECULAR CHARACTERIZATION OF A BIFUNCTIONAL ENZYME (DEHYDROQUINATE DEHYDRATASE/SHIKIMATE DEHYDROGENASE) AND THE POST-PREPHENATE PATHWAY ENZYMES NEEDED FOR PHENYLALANINE AND TYROSINE BIOSYNTHESIS IN *NICOTIANA* SPP.

BY

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ADH	arogenate dehydrogenase
ADT	arogenate dehydratase
AGN	*L*-arogenate
αKG	α-ketoglutarate
AMP	ampicillin
ANS	anthranilate synthase
ANS-1	aneuploid *N. silvestris* suspension cell line
βME	beta mercaptoethanol
BSA	bovine serum albumin
CHA	chorismate
CM	chorismate mutase
CM-52	carboxymethyl cellulose-52 cation exchange column
CM-I	chloroplast chorismate mutase
CM-II	cytosolic chorismate mutase
DAHP	3-deoxy-*D*-*arabino*-heptulosonate 7-phosphate
DAHPS	DAHP synthase, enzyme 1 of aromatic pathway
DE52	(DEAE) diethylaminoethyl cellulose-52 anion exchange column
DHQ	dehydroquinate
DHS	dehydroshikimate
DQT	dehydroquinate dehydratase or dehydroquinase
Ds-Co	cytosolic DAHP synthase of plants
Ds-Mn	chloroplast DAHP synthase of plants
DTT	dithiothreitol
E-cells	cells of about two generations of exponential growth
EDTA	ethylenediaminetetraacetate
EE-cells	cells in continuous exponential growth
EPPS	(N-[2-Hydroxyethyl]piperazine-N'-[3-propanesulfonic acid];HPPS)
EPSP	5-*enol*pyruvylshikimate-3-phosphate
E4P	erythrose 4-phosphate
HA	hydroxylapetite column
HPLC	high performance liquid chromatography
HPP	4-hydroxyphenylpyruvate
ICBR	Interdisciplinary Center of Biotechnology Research
IPTG	isopropylthio-β-*D*-galactoside
KPO_4	potassium phosphate buffer
LB	Luria-Bertaini medium
LB-AMP	LB plus ampicillin
LES	lag, exponential and stationary phases of growth
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; (thiazolyl blue)
NADP	nicotinamide adenine dinucleotide
NTPS	nucleotide triphosphates

OAA	oxaloacetate
OPA	orthopthalaldyhyde
PAGE	polyacrylamide gel electrophoresis
PAT	prephenate aminotransferase
PCA	protocatechuic acid
PCMB	p-chloromercuribenzoate
PDH	prephenate dehydrogenase
PDT	prephenate dehydratase
PEG_{8000}	polyethylene glycol
PEP	phosphoenolpyruvate
PLP	pyridoxal 5'-phosphate
PMS	N-methyldibenzopyrazine methylsulfate (phenazine metasulfate)
PMSF	phenylmethylsulfonyl fluoride
PPA	prephenate
PPY	phenylpyruvate
PCA	protocatechuic acid
QA	quinic acid
QDH	quinate dehydrogenase
QDT	quinate dehydratase
RE	restriction enzymes
SA	specific activity
S-protein	shikimate-bifunctional DQT/SDH protein
SDH	shikimate dehydrogenase
SDS	sodium dodecyl sulfate
SP-I	(S1-protein) bifunctional protein (DQT/SDH), major peak from Celite 545 chromatography separation
SP-II	(S2-protein) bifunctional protein (DQT/SDH), minor peak from Celite 545 chromatography separation
SHK	shikimate

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

MOLECULAR CHARACTERIZATION OF A BIFUNCTIONAL ENZYME (DEHYDROQUINATE DEHYDRATASE/SHIKIMATE DEHYDROGENASE) AND THE POST-PREPHENATE PATHWAY ENZYMES NEEDED FOR PHENYLALANINE AND TYROSINE BIOSYNTHESIS IN *NICOTIANA* SPP.

By

Carol Ann Bonner

April 1994

Chairman: Roy A. Jensen
Major Department: Microbiology and Cell Science

Two bifunctional proteins containing catalytic domains for dehydroquinate dehydratase (dehydroquinase) and shikimate dehydrogenase were separated from extracts of *Nicotiana silvestris* suspension cells by use of a decreasing ammonium sulfate gradient on a Celite 545 column. The two apparent isoenzymes, denoted as SP-I and SP-II, were purified 1000- and 800-fold, respectively. Higher molecular mass values were obtained for SP-II than for SP-I on the criteria of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and gel filtration chromatography. Each protein exhibited multiple bands on SDS-PAGE and on native-PAGE monitored with a specific shikimate

dehydrogenase activity stain. Temperature and pH optima for catalysis were similar for each protein, as were the temperatures of inactivation. However, Km values for shikimate were 0.80 and 0.36 m*M* for SP-I and SP-II, respectively. Antibody raised against SP-I cross-reacted with SP-II, but not with *Escherichia coli* proteins possessing the corresponding activities. Antibody screening of a cDNA library of *Nicotiana tabacum* was used to isolate clones expressing the S-protein. Functional complementation of *aroD* and *aroE E. coli* auxotrophs transformed with plasmids carrying cloned cDNA were successful. Activities for each enzyme were 15-fold greater in the transformed mutant strains than in the wild type strain of *E. coli*. Analysis of the amino acid sequence deduced from the cloned cDNA sequence revealed homology with the appropriate functional domains of the pentafunctional Aro1 protein of *Saccharomyces cerevisiae* and with the monofunctional AroD and AroE proteins of *E. coli*. The post-prephenate pathway enzymes were also studied in *Nicotiana silvestris* suspension cells. Levels of arogenate dehydratase activity changed throughout a growth cycle in suspension cells. Using specific antibodies, several putative cDNA clones encoding prephenate aminotransferase or arogenate dehydrogenase were isolated for further study. The base of comparative enzymology in higher plants for the aforementioned enzymes was extended to the unicellular alga, *Chlorella sorokiniana*.

Prephenate aminotransferase of *Chlorella* exhibited the striking high-temperature activity optimum and specificity for prephenate that is typical of higher plants.

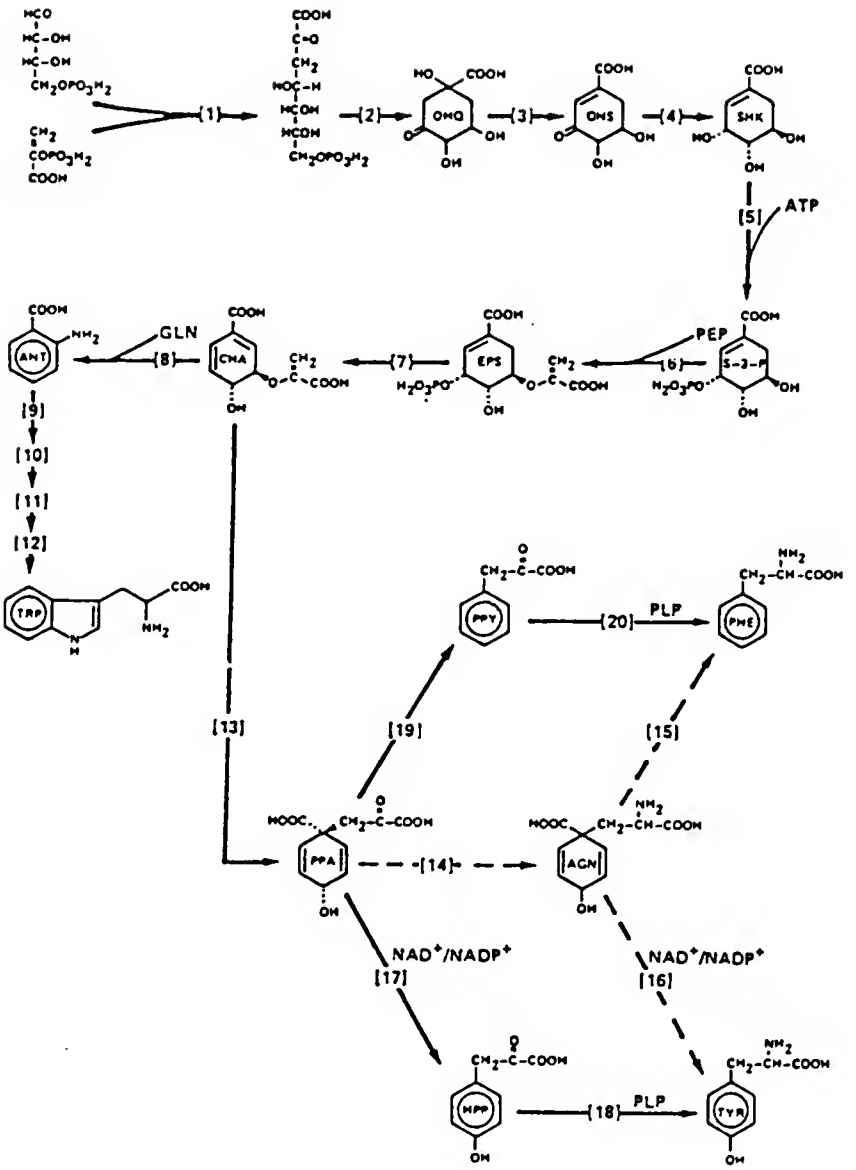
CHAPTER I

LITERATURE REVIEW AND RATIONALE

Aromatic Amino Acid Biosynthesis in Plants

In higher plants, the three aromatic amino acids (*L*-phenylalanine, *L*-tyrosine and *L*-tryptophan) are not only required for protein synthesis and primary metabolite synthesis (such as the hormone, indole acetic acid and the structural component of woody plants, lignin), but also must be available as starting substrates for a vast array of secondary metabolites including alkaloids, coumarins, isoflavones, and tannins (2, 78, 79). Intermediates of the pathway are also precursors for synthesis of other essential metabolites in plant cells. The vitamin-like derivatives, folic acid and ubiquinone, are synthesized from chorismate, the first branch point intermediate in the aromatic pathway. Protocatechuic acid is derived from the common pathway intermediate, dehydroshikimate (Fig. 1). It has been estimated that up to 60% of plant carbon flows through the aromatic amino acid pathway (40). Humans as well as other higher forms of life depend heavily on plants to provide the three essential aromatic amino acids, and knowledge of aromatic biosynthesis in higher plants is fundamentally

Fig. 1-1. Aromatic amino acid biosynthetic pathway. The common portion of the biosynthetic pathway leading to the branch point intermediate, chorismate, begins with DAHP synthase [1] catalizing the condensation of erythrose 4-phosphate and phosphoenolpyruvate and consists of seven steps. These steps consist of dehydroquinate synthase [2], dehydroquinate dehydratase [3], shikimate dehydrogenase [4], shikimate kinase [5], EPSP synthase [6], and chorismate synthase [7]. Chorismate, at the branchpoint of the pathway is utilized in one direction to yield *L*-tryptophan (steps 8-12) and in another direction by chorismate mutase [13] to produce prephenate, another branch point intermediate. Organisms in nature use different routings from prephenate. Higher plant chloroplasts proceed through prephenate aminotransferase [14] and arogenate dehydratase [15] to form PHE, or through prephenate aminotransferase and arogenate dehydrogenase [16] to form TYR. In other organisms a second route proceeds through prephenate dehydratase [19], then through aromatic (or PHE) aminotransferase [20] to synthesize PHE, or through prephenate dehydrogenase [17] and through aromatic (or TYR) aminotransferase [18] to synthesize TYR.


[1]
[2]
DHQ
[3]
DHS
[4]
SHK
[5]
ATP
S-3-P
PEP
[6]
EPS
[7]
CHA
GLN
[8]
ANT
[9]
[10]
[11]
[12]
TRP
[13]
PPY
[20]
PLP
PHE
[19]
[15]
PPA
[14]
ACN
NAD+/NADP+
[17]
NAD+/NADP+
[16]
HPP
[18]
PLP
TYR

important. Figure 1 shows a composite schematic of the biosynthetic steps of the common portion of the pathway and of the three divergent amino acid branches.

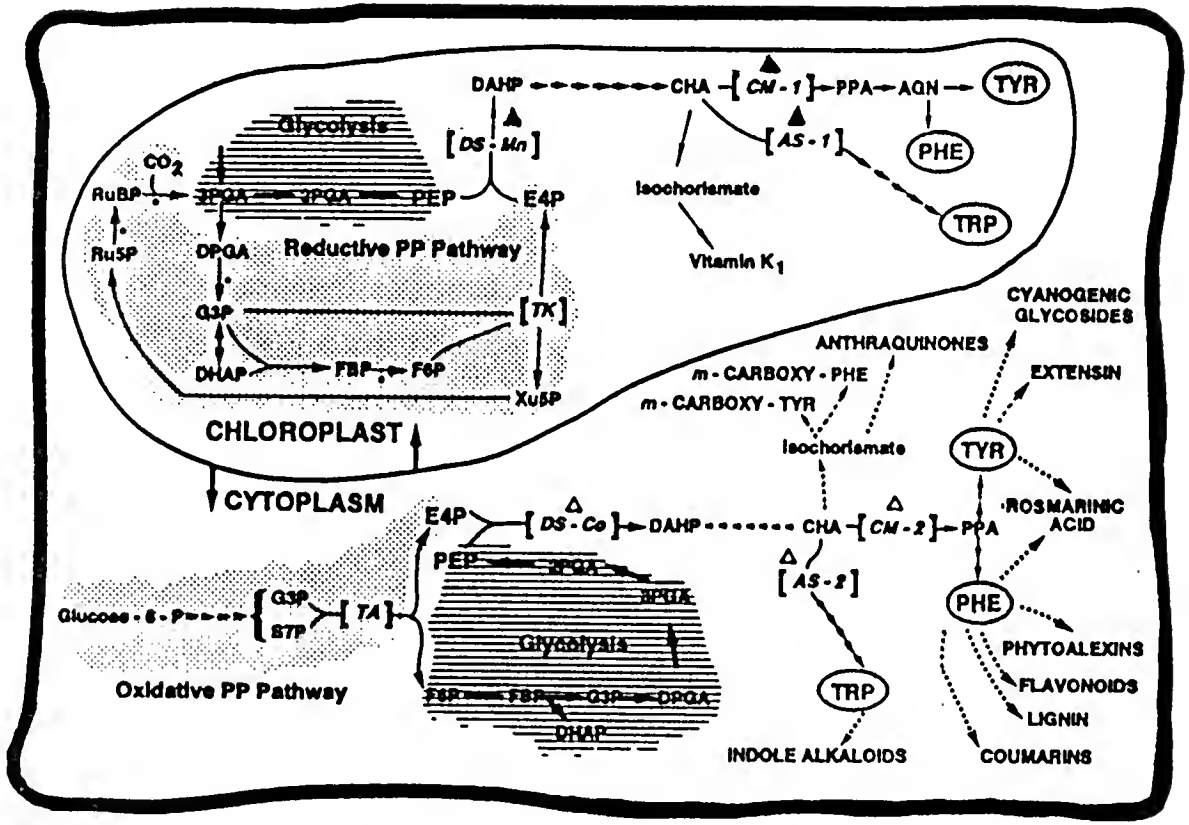
It has been established that a complete aromatic biosynthetic pathway is present in the chloroplasts of plant cells (2, 41). The common portion of the pathway begins with the condensation of erythrose 4-phosphate and phosphoenol pyruvate to form 3-deoxy-*D*-*arabino*-heptulosonate 7-phosphate by DAHP synthase and continues with six more enzymatic steps to form CHA, the major branchpoint intermediate. From CHA, the pathway branches to form *L*-tryptophan via anthranilate synthase (ANS, enzyme 8) and to *L*-phenylalanine or *L*-tyrosine via chorismate mutase (CM, enzyme 13). From prephenate, the product of the CM reaction, different pathway variations have been shown in nature to complete the synthesis of PHE and TYR (16). In plant chloroplasts, the post-prephenate pathway begins with the transamination of PPA to yield *L*-arogenate by prephenate aminotransferase (PAT, enzyme 14). Finally, AGN is either converted to PHE by arogenate dehydratase (ADT, enzyme 15) or to TYR by arogenate dehydrogenase (ADH, enzyme 16). This pathway is illustrated with dashed arrows in Fig. 1.

The basic hypothesis underlying my studies is that a second pathway for aromatic amino acid biosynthesis exists in the cytosol compartment of higher plants. The supportive evidence is that DAHP synthase (the early-pathway enzyme)

(32), CM (the mid-pathway enzyme) (23) and ANS (the initial TRP-branch enzyme) (15) are present as pairs of separately compartmented isoenzymes (Fig. 1-2). It is also known that starting substrates for aromatic biosynthesis (E4P and PEP) are synthesized by duplicate pathways of carbohydrate metabolism located in both the plastid and cytosolic compartments (Fig. 1-2). Since most secondary metabolites are synthesized in the cytosol of plant cells (39) (Fig. 1-2), it seems likely that an aromatic pathway should be available in the cytosol to produce aromatic amino acids not only for protein synthesis but as starting substrates for secondary metabolism as well.

Cytosolic isoenzymes of DAHP synthase and chorismate mutase (Ds-Co and CM-II, respectively) are markedly different from Ds-Mn and CM-I (the corresponding plastid-localized isoenzymes) in both catalytic and regulatory properties (32, 23). Such differences and indeed a lack of homology would be consistent with the endosymbiotic hypothesis of organelle evolution (55). If cytosolic species of other pathway steps are present in plant cells, it would not be surprising to find them to have equally divergent properties that reflect their xenologous origin at the time of endosymbiosis. Thus, the alternative post-prephenate steps shown in Fig. 1 (solid arrows) might be used in the cytosol. From this perspective it seems unlikely that dehydroquinase and shikimate dehydrogenase

Fig. 1-2. Multiple compartmentation of pathways in plant cells. Glycolysis and the pentose phosphate pathways are shown in both the chloroplast and the cytosol compartments of higher plants. An intact aromatic amino acid pathway is shown in the chloroplast. A hypothetical aromatic amino acid pathway is shown in the cytosol. Three chloroplast isoenzymes of aromatic biosynthesis (▲) and the corresponding cytosolic isoenzymes (△) are shown. Numerous metabolites, known to be synthesized in the cytosol from aromatic amino acids, PHE, TYR and TRP, are given.


DAHP
CHA
[CM-1]
PPA
AGN
TYR
PHE
TRP
[DS-Mn]
Glycolysis
CO2
RuBP
3PGA
1,3PGA
PEP
E4P
Ru5P
DPGA
Reductive PP Pathway
G3P
[TK]
DHAP
FBP
F6P
Xu5P
[AS-1]
Isochorismate
Vitamin K1
CHLOROPLAST
CYTOPLASM
CYANOGENIC GLYCOSIDES
ANTHRAQUINONES
EXTENSIN
m-CARBOXY-PHE
m-CARBOXY-TYR
Isochorismate
TYR
ROSMARINIC ACID
[DS-Co]
DAHP
CHA
[CM-2]
PPA
[AS-2]
PHE
PHYTOALEXINS
FLAVONOIDS
LIGNIN
COUMARINS
Glucose-6-P
G3P
E4P
[TA]
Glycolysis
Oxidative PP Pathway
TRP
INDOLE ALKALOIDS

would co-exist as a bifunctional protein, as they do in plastids. Alternative possibilities would be that they are monofunctional species, or that they are domains of a large pentafunctional protein like that present in fungi (21).

That the enzymes catalyzing the third and fourth steps in the common pathway of plastids are bifunctional in higher plants has been documented (14, 61). In *E. coli*, DQT and SDH are each found as monofunctional proteins (18,19). Yeast and fungi produce the arom protein (21), a pentafunctional protein bearing functional domains corresponding to steps 2 through 6 of aromatic amino acid biosynthesis (Fig. 1-1). It is interesting that when the arom pentafunctional protein of *Neurospora crassa* is subjected to limited proteolysis, the DQT/SDH domains were retained as an intact and functional fragment (77).

Rationale for Focus upon DQT and SDH

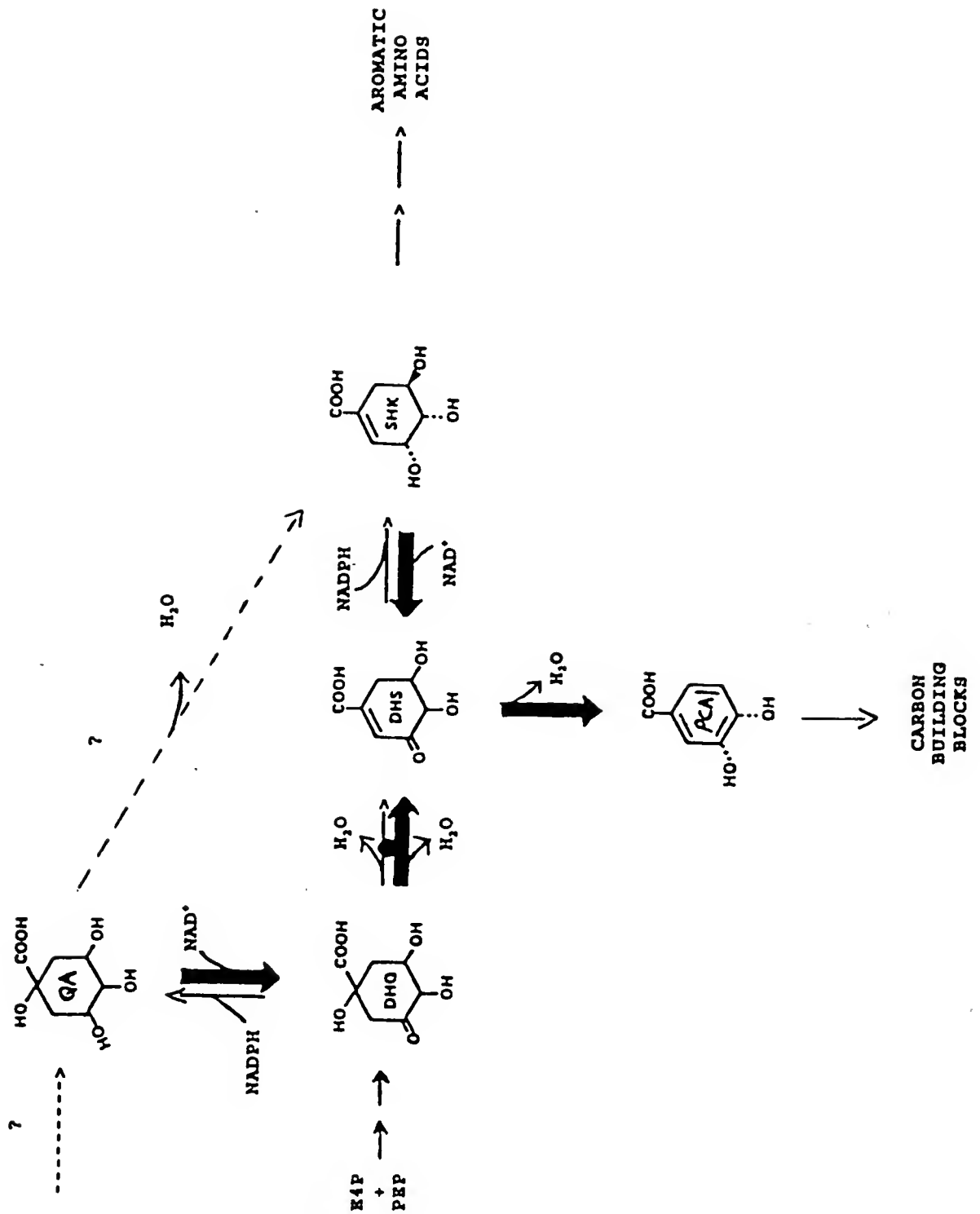
The rationale for choice of the DQT/SDH protein (or S-protein) for study was as follows. (i) SDH is easily assayed, has very high activity and the substrates are readily available. (ii) The S-protein is in the middle of the common portion of the pathway and provides a link from the early-pathway isoenzyme pair of DAHP synthase to the second known mid-pathway pair of isoenzymes of chorismate mutase. (iii) Two separable shikimate dehydrogenases had been demonstrated by Rothe et al. (67) in pea seedlings, and

my preliminary experiments had shown this result to be repeatable in *N. silvestris*. (iv) Distinctly different properties of the isoenzyme pairs for DAHP synthase, chorismate mutase, and anthranilate synthase have been most useful to demonstrate the separate spatial locations. Since DQT and SDH were known to coexist as a plastid-localized bifunctional protein, I thought it likely that the corresponding cytosolic enzymes would be monofunctional. If so, this would be a type of differential property that might allow me to distinguish the two during cell fractionation studies.

Shikimate biosynthesis and quinate catabolism

In monocots, such as *Zea mays*, there are two dehydroquinases, one is bifunctional with SDH (specific for $NADP^+$) and the other is either bifunctional or stably complexed with a NAD^+ specific quinate dehydrogenase (36). The pathway steps to quinic acid in plants have not yet been rigorously established and may originate from E4P and PEP via DHQ, or from unknown pathways. Quinate has been shown to accumulate in plant vacuoles and to account for at least 10%, if not more, of the dry weight of certain plant tissues (2, V. I. Ossipov, personal communications). The quinate catabolic system from fungi (34,35) overlaps the biosynthetic shikimate pathway with several analogous dehydrogenase and dehydratase reactions (Fig. 1-3). An

Fig. 1-3. Quinate catabolism and aromatic amino acid biosynthesis. Aromatic amino acid biosynthesis is shown by the thin horizontal arrows. The quinate catabolic system is shown by the heavy arrows starting with NAD^+ specific quinate dehydrogenase, dehydroquinate dehydratase and dehydroshikimate dehydratase to form protocatechuic acid. The remaining heavy arrow is the NAD^+/QDH which also uses SHK. The heavy dot between the thin and thick arrow emphasizes the overlapping step common to biosynthesis and catabolism, ie, the biosynthetic dehydroquinase and the inducible catabolic dehydroquinase. Biosynthetic NADPH specific QDH (the thin vertical arrow) may use DHQ to synthesize QA, or may be used in an unknown route for QA biosynthesis shown by the short dashed arrow. The long dashed arrow show quinate dehydratase, an activity reported in some plants.


AROMATIC AMINO ACIDS
SHK
NADPH
NAD+
DHS
H2O
ACAI
CARBON BUILDING BLOCKS
QA
NAD+
NADPH
DHQ
H2O
H2O
?
H2O
?
E4P
+
PEP

inducible catabolic dehydroquinase takes DHQ that comes directly from quinic acid (by a specific NAD^+ quinate dehydrogenase) to dehydroshikimate which is converted to PCA and on to carbon building blocks generated by the TCA cycle. The dehydroquinase functional domain of the pentafunctional arom protein of yeast and fungi channels DHQ formed from DAHP to EPSP, thus bypassing PCA formation and avoiding futile cycling (17, 21, 28). Both a NADPH specific QDH (V. I. Ossipov, personal communication) and a quinate dehydratase (V.I. Ossipov, personal communication) have recently been shown. The NAD^+ specific QDH from *E. nidulans* also will utilize SHK (34). These two systems offer some interesting possibilities for overlapping metabolism in plants. For example, it is conceivable that a QDT might convert quinate to SHK for synthesis of aromatic amino acids (Fig. 1-3) to be utilized as precursors for the synthesis of lignin and other compounds.

Multiple Species of Shikimate Dehydrogenase

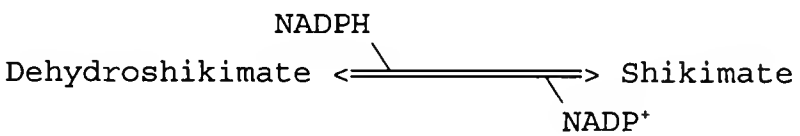
Shikimate dehydrogenase has been a popular enzyme choice for a variety of isoenzyme studies and multiple activities have been described on many occasions.

The bifunctional DQT/SDH protein ($NADP^+$ specific) was examined in dicots (50). No evidence of a separate DQT or SDH has been determined, thus far.

Cofactor specificity. Rothe et al. (67) separated two SDH ($NADP^+$) activities from pea seedlings on a Celite 545 column with an ammonium sulfate gradient. Whether DQT activity coeluted with one or both of the SDH activities was not examined. Shikimate dehydrogenase was shown to be located in the stroma of spinach chloroplasts and was partially purified (30). Mousdale et al. (61) purified a bifunctional DQT/SDH ($NADP^+$ specific) from pea seedling chloroplasts and also showed by chromatofocusing that there were two chloroplast isoenzymes and one unstable isoenzyme, probably located in the cytosol. Two forms of nondissociable DQT/SDH ($NADP^+$) activities and one of QDH (NAD^+) were found in *Phaseolus mungo* seedlings by Minamikawa (58). Koshiba (49) also found two forms of DQT/SDH ($NADP^+$) activities from *Phaseolus mungo* seedlings. In 1988, Ogawa and Tateoka (64) reported one SDH ($NADP^+$) specific activity and two SDH (NAD^+) specific activities (DQT apparently was not assayed in these fractions) from *Phaseolus mungo*. This is the first description of NAD^+ specific SDH activities and is surprising since the same methodology for separation was used by the three groups and each checked for specificity of cofactors. A recent study of QDH in *Phaseolus mungo* by Kang and Scheibe (44) found that the enzyme could use $NADP^+$ as well as NAD^+. When SHK was added to the reaction mix containing quinic acid and NAD^+, some inhibition was found. Apparently SHK was not tried as a substrate for QDH. If QDH

also functions as a SDH, competition for two products (dehydroquinate and dehydroshikimate) would occur and this could account for the inhibition seen with SHK added to the reaction mix. This might also explain the earlier report of the NAD^+ specific SDH activity by Ogawa and Tateoka (64), if the enzyme was actually a nonspecific QDH. The *qa* gene cluster of *Neurospora crassa* encodes a functional domain for a QDH (NADH specific) that will also utilize SHK/NADH (34).

Properties. The range of molecular mass for DQT/SDH (or SDH, where DQT was not studied) is from 44,000 to 73,000. It is not clear as to whether the lower M_r of 44,000 might represent a monofunctional SDH. SDH activity is usually assayed in the reverse of the physiological direction with the substrates, SHK and $NADP^+$, rather than the forward direction with DHS and NADPH:



Values of Km have been determined and vary from 0.060 to 1.3 m*M* for SHK, from 0.010 to 0.10 for $NADP^+$, and from 0.130 to 0.180 m*M* DHQ. Table 1-1 gives estimates of M_r and Km values for DQT and SDH from several plant species, as well as M_r from *E. coli* and from *S. cerevisiae*.

Inhibition studies with $HgCl_2$, p-chloromercuri benzoate and oxidized glutathione have suggested that SDH

Table 1-1. Molecular mass and Km values of DQT and SDH.

Source	Substrate	Km (m*M*)	M_r[a]	Reference
Sweet potato	SHK	1.300	--	48
	NADP	0.100		
Mung bean 1	DHQ	0.130	57,000 (GE)	49
	SHK	0.220		
	NADP	0.025		
Mung bean 2	DHQ	0.180	57,000 (GE)	49
	SHK	0.910		
	NADP	0.025		
Tomato	SHK	0.038	73,000 (GF)	52
Pea	SHK	0.690	59,000	61
	NADP	0.013		
Corn 1		--	55,000 (GE)	13
Corn 2		--	49,000 (GE)	
Spinach		--	67,000 (GF)	30
			59,000 (GE)	
Cucumber	SHK	0.060	44,000 (GF)	53
	NADP	0.010		
Radish	SHK	0.860	60,000 (GF)	71
	NADP	0.090		
Spinach	SHK	0.480	44,000 (GF)	71
	NADP	0.032	49,000 (GE)	
E. coli		--	56,000 (AA)	28[b]
S. cerevisiae		--	68,000 (AA)	77

[a]Molecular mass estimated by gel filtration (GF), by PAGE (GE) or calculated from the deduced amino acid sequence (AA).
[b]Molecular mass of monofunctional DQT and SDH were combined.

sulfhydryl groups act as functional groups in the catalysis of the enzyme and that partial reversal of inhibition is accomplished by thiols (48, 53). Aromatic compounds, metals, and salts have been studied with respect to effect on DQT and SDH activities. Protocatechuic acid was found to be a competitive inhibitor of SDH activity in tomato (52,) and Cl^- inhibited DQT activity competitively in pea (61). Ca^{2+} stimulated SDH activity in *Brassica rapa* (71).

Molecular genetics. Plant genes encoding DQT or SDH have not been sequenced, but the monocistronic *aroD* and *aroE* genes have been cloned and sequenced in *E. coli* (1, 27). Amino acid sequences are also available for discrete domains encoding these activities in *S. cerevisiae* (28) and *Aspergillus nidulans* (now known as *Emericella nidulans*) (17) as part of the pentafunctional arom gene (*aroM*). The *E. coli* amino acid sequences, *aroD* (DQT) and *aroE* (SDH) exhibit only have about 25 and 21% identity, respectively, with the corresponding functional domains of the yeast arom protein.

In higher plants, aromatic-pathway genes thus far cloned and sequenced are limited to DAHP synthase (29), shikimate kinase (70), EPSP synthase (73), and chorismate synthase (69) of the common portion of the pathway. Anthranilate synthase (63) and tryptophan synthase β subunit (3) of the tryptophan branch of the pathway have been cloned and sequenced.

The Post-Prephenate Pathway in Plants

The dual-pathway hypothesis of aromatic amino acid biosynthesis specifies that an intact post-prephenate pathway exists in the cytosol in addition to that already demonstrated in the plastid compartment (41). If a separate cytosolic pathway functions: (i) separate genes encoding isoenzyme counterparts of the plastid-localized enzymes may exist, (ii) separate genes encoding alternative enzymes

steps may exist, or (iii) the unprocessed preprotein precursor of the plastid enzymes may function in the cytosol.

Prephenate aminotransferase. PAT of higher plants was first characterized in partially purified extracts, separated from aromatic aminotransferases, and shown to be unusually specific for its substrate, prephenate. Activity was optimal at 70°C for PAT, a temperature which inactivated other interfering aminotransferases (5). PAT was purified about 1000-fold (7), and localization studies showed the enzyme to be located in the chloroplast (Bonner and Jensen, unpublished data, 1985; 41, 76). A simple, inexpensive spectrophotometric assay was developed for PAT by following the increase in oxaloacetate when the *L*-aspartate/α-ketoglutarate couple was used (6). In the same study, the ASP/αKG couple was used with native PAGE activity stained gels to relate the position of PAT with respect to other aminotransferases and to show the disappearance of heat-inactivated aminotransferases.

Arogenate dehydrogenase. Partially purified ADH was studied in *N. silvestris* with respect to allosteric regulation by TYR by Gaines et al. (31). ADH was partially purified and regulatory properties were determined in *Sorghum bicolor* (22). The enzyme was purified about 1000-fold (8). PAT, ADH and SDH activities were followed throughout a growth cycle in *N. silvestris* suspension cells

(12) and after wounding of potato (59).

Arogenate dehydratase. ADT activity was first determined in *N. silvestris* cells by Jung et al. (43). The enzyme was localized in spinach chloroplasts and allosteric regulation (activation by TYR and inhibition by PHE) was studied (43). Siehl and Conn (75) partially purified ADT from *Sorghum bicolor* and studied some kinetic and regulatory properties.

Rationale for focus upon the post-prephenate enzymes. The rationale in choosing to study some aspects of the post-prephenate enzymes for this dissertation is my long-term objective to follow up earlier work with molecular-genetic approaches. Studies of the individual enzymes may shed some light as to the multiplicity of compartmentation. The merits are as follows. A) Prephenate aminotransferase (i) has been characterized and a well-developed HPLC assay exists (5), (ii) has important biotechnological application due to its unique aminotransferase properties and the value of its product, *L*-arogenate, and (iii) can be purified by an established procedure (7). B) Arogenate dehydrogenase (i) has an established purification methodology (8) for providing purified protein for antibody production that may be used to probe cDNA libraries, and (ii) is easily assayed fluorometrically. C) Arogenate dehydratase has an established HPLC assay (43), but is by far the most difficult of the three enzymes to assay and purify.

CHAPTER II

MATERIALS AND METHODS

Organisms and extract preparations

Eukaryotes

Nicotiana silvestris Speg. et Comes suspension cultured cells originally isolated from haploid leaf material have been maintained in our laboratory for about twelve years and are well described physiologically and biochemically (10, 11, 12). Cell populations are subcultured in stationary phase (7 day), diluted fivefold into fresh Murashige and Skoog medium (62), and grown under specified conditions in controlled growth chambers (10). For these experiments (unless otherwise stated) seven-day old cells were harvested by filtering through Miracloth (Calbiochem, LaJolla, CA), rinsed with nanopure water, ground in liquid nitrogen in a Waring blender and stored at -70^{0}C. until use.

C. sorokiniana cells cultured as described by Meridith and Schmidt (57) were kindly provided by Dr. Robert Schmidt.

Bacterial strains and plasmids

E. coli strains used for transformation include DH5α, AB1360 *aroD* (DQT^{-}) and SK494 *aroE* (SDH^{-}). Plasmids used for subcloning were pUC18, pGEM5Zf(+) or pBluescript SK+.

Preparation of *N. silvestris* extract

Crude extracts for assay. Frozen, ground *N. silvestris* cells were suspended (1:1) in 100 m*M* KPO_4 buffer at pH 7.3 containing 20% glycerol, 1.0 m*M* phenylmethylsulfonyl fluoride and 1.0 m*M* dithiothreitol, centrifuged at 35,000 x g at 4°C for 20 min. Extracts were treated to 80% of saturation with ammonium sulfate, centrifuged as above, followed by resuspension of pellets in EPPS-KOH buffer (pH 8.6) with all of the above components and dialyzed with four changes of buffer until ammonia could not be detected by HPLC. Extracts not treated with ammonium sulfate were desalted using PD10 columns in Epps-KOH buffer as above or dialyzed overnight with 4 changes of the Epps-KOH buffer.

Extracts for purification of ADH and PAT. Five-day *N. silvestris* suspension cells were used in the purification of ADH and PAT. Crude extracts were prepared as described above and also included 0.1 m*M* pyridoxal 5' phosphate and 1.0 m*M* EDTA in order to stabilize PAT (5).

Extracts for S-protein purification. For purification of the S-proteins, frozen, ground seven-day *N. silvestris* suspension cells (300 g) were suspended into 300 ml of 100 m*M* KPO_4 buffer, pH 7.8, containing 20% glycerol, 1.0 m*M* DTT, 1.0 m*M* benzamidine, 0.2 m*M* PMSF and 0.05 m*M* leupeptin. A sample was immediately desalted using a PD10 column,

equilibrated with 50 m*M* EPPS-KOH buffer at pH 8.6, containing the same components as above and labeled as crude extract.

Preparation of cultured cells for assay of ADH levels

The isogenic suspension cell-culture line of *N. silvestris* was grown in continuous light with gentle shaking at 150 rev/min. The growth properties of a *N. silvestris* suspension cell line (ANS-1) in lag, exponential, and stationary phases of growth have been characterized throughout a conventional growth cycle (referred to as E cells) and for cells which have been maintained in exponential growth for more than 10 generations, EE cells (10). For routine subculture, 80 ml of suspension-culture medium were inoculated with 20 ml of 7-day stationary-phase cells in 500-ml Erlenmeyer flasks. EE cells (2 g wet weight) were transferred to fresh medium on day 7 when cells reached about 1 X 10^6 cells ml^{-1}. Daily samples were harvested using Miracloth for filtration, washed with distilled water, ground and frozen in liquid nitrogen, and stored at -70^{0}C until extract preparation. Daily sampling was also done to determine a cell count by mixing 1 ml of a 50 ml culture with 12% chromium trioxide (helps to separate cell clumps and visualize cells more easily). Samples were heated to 70^{0}C for 2 to 5 min before counting in a Fuchs-Rosenthal counting chamber at 20x magnification. The remaining 49 ml of culture were filtered and cells were

dried on preweighed filters made from Miracloth in an oven at 50°C and were weighed daily until constant dry weight values were obtained. From these two methods, growth curves were determined.

Preparation of *C. sorokiniana* extracts

Frozen cell pellets of *C. sorokiniana* were suspended into 50 m*M* PIPES buffer, pH 7.3, containing 1.0 m*M* DTT, 1.0 m*M* PMSF, 35 m*M* KCl, and were broken by 2 passes through a French Pressure Cell at 20,000 psi. The slurry was centrifuged at 18,000 x g, at 4°C for 15 min, and the supernatant was next centrifuged at 150,000 x g for 1 h at 4°C. Extracts were either desalted and used for column chromatography, or dialyzed with 50 m*M* EPPS-KOH buffer with all protective components as listed above for use in enzyme assays. (Pipes buffer plus KCl was used in place of EPPS buffer for column chromatography in order to separate isoenzymes of chorismate mutase for another study.) Glycerol and PLP were added to column fractions to protect several of the enzymes to be assayed (PAT and ADH).

Preparation of bacterial extracts

The mutant *E. coli* strains (*aroD* and *aroE*) carrying the cDNA clones encoding functional proteins (DQT or SDH, respectively) were grown in 200 ml of LB/Amp media, washed and resuspended in KPO_4 buffer containing 20% glycerol and 1 m*M* DTT at pH 7.6, and centrifuged at 150,000 x g, at 4°C for

1 h. The supernatants were desalted by passage through Sephadex G-25 columns before fluorometric assay.

Enzyme assays

Aminotransferases

Prephenate aminotransferase. Prephenate aminotransferase was usually incubated with concentrations of 20 m*M* *L*-glutamate and 0.8 m*M* prephenate (unless otherwise stated), incubated at 37°C for 10 min (or as otherwise stated) and then assayed by HPLC as an orthopthalaldyhyde derivative in a 60% methanol/40% of 20 m*M* KPO_4 buffer system. Peaks of PHE were quantified before and after acidification of the product of the reaction, AGN, in 1*N* HCl for 10 min at 37°C.

Aminotransferase couples. Other aminotransferase assays included an amino acid donor (ASP, TYR, LEU or ALA) and keto acid (PPY) at concentrations as stated and were assayed similarly by HPLC, before and after acidification when applicable. Controls included samples without amino acid donor or keto acid.

Arogenate dehydratase

Arogenate dehydratase was assayed according to Jung et al. (43) by measurement of OPA derivatives following HPLC where the peak of AGN formed were monitored before and after acidification to PHE. Concentration of 0.5 m*M* AGN, 0.25 m*M* TYR (activator), 50 m*M* EPPS-KOH buffer at pH 8.6 and enzyme

were included in the reaction sample of 100 μl that were incubated at 32°C for 30 min. Controls included samples without AGN or enzyme.

Dehydroquinase

Dehydroquinase activity was assayed spectrophotometrically at 234 nm at room temperature (24°C) by following the rate of increase of product, dehydroshikimate, when dehydroquinate was provided as substrate. Unless otherwise stated, a reaction sample included 0.5 m*M* DHQ, enzyme and 50 m*M* EPPS-KOH buffer at pH 8.6. An extinction coefficient of 12,000 was used to calculate activity (37). DQT was also assayed fluorometrically via a coupled assay with SDH. DHS formed from DHQ by the dehydratase reaction was reduced with NADPH (cofactor for SDH) and the rate of NADPH disappearance was followed. Reaction samples contained 0.5 m*M* DHQ, 0.2 m*M* NADPH in 50 m*M* EPPS-KOH at pH 8.6 for column fractions or 50 m*M* Bis-tris propane buffer, pH 7.5, for other studies as stated. Controls were carried out for each enzyme assayed by adding only one substrate for about three to five min before adding the second substrate.

Dehydrogenases

Dehydrogenase reactions were usually assayed fluorometrically at room temperature (24°C) with cofactor $NAD(P)^+$ for 1 to 2 min before addition of substrate, and the rate of formation of NAD(P)H was monitored (340 nm emission

and 460 nm excitation). Unless otherwise stated, a total vol of 400 μl contained substrates and 50 m*M* EPPS-KOH buffer at pH 8.6. The rate is expressed as fluorometric units per min (FU min^{-1}). Activities were calculated as nmol min^{-1} by relating experimental values to a standard curve obtained with authentic NADPH. Any background rate seen when cofactor was present alone in reaction sample (only in some crude extracts) was subtracted from the rate obtained from reaction sample containing both substrates to give an appropriate correction. A spectrophotometric assay was also used occasionally by following the rate of formation of NAD(P)H at $A_{340\ nm}$.

Arogenate dehydrogenase. Arogenate dehydrogenase activity was determined when 0.5 m*M* AGN was added to the reaction assay after 0.5 m*M* $NADP^+$ had been added or as otherwise stated. When appropriate, NAD^+ was added as cofactor to the reaction mix.

Prephenate dehydrogenase. Prephenate dehydrogenase activity was assayed with 0.5 m*M* $NADP^+$ (or NAD^+) and 1.0 m*M* PPA.

Shikimate dehydrogenase. Shikimate dehydrogenase activity was usually assayed in the reverse-of-physiological direction with 1.0 m*M* $NADP^+$ and 4.0 m*M* SHK, unless otherwise stated. When appropriate, NAD^+ was added to the reaction sample. In the forward direction, SDH was assayed with

concentrations of 1.0 m*M* NADPH and 1.0 m*M* DHS or at concentrations otherwise stated.

Quinate dehydrogenase. Quinate dehydrogenase was assayed with 1.0 m*M* quinate and 1.0 m*M* NAD^+ or $NADP^+$ in 100 m*M* glycine buffer at pH 9.5.

Prephenate dehydratase

Prephenate dehydratase was assayed by incubation of PPA and enzyme in 50 m*M* EPPS buffer (total vol of 100 μl) at 37°C for 10 to 20 min before addition of 100 μl 1N HCl followed by further incubation at 37°C for 10 min. Controls included PPA or enzyme with buffer only. Activity was calculated from spectrophotometric readings at $A_{320\ nm}$ and an extinction coefficient of 17,500.

Protein estimations

Protein concentrations were estimated by the method of Bradford (14) with BSA as a standard. A micro-assay was used, as described, to estimate highly purified protein concentrations.

Definition of activity and specific activity

Activity for all enzyme reactions in this study are defined as nmol min^{-1} for the stated amount of protein added into a standard assay volume. Specific activity is defined as nmol min^{-1} mg $protein^{-1}$.

Separation of pathway enzymes by chromatography

Column chromatography of *N. silvestris* extracts

A DEAE-cellulose column for separation of enzymes of aromatic biosynthesis was prepared similarly as described under purification. A 100 mg amount of protein was applied to a bed volume of 102 ml of a DEAE column equilibrated in 50 m*M* KPO_4 at pH 7.5. After washing the column with about 3 column volumes of buffer, a 600 ml gradient from 0 to 0.4 *M* KCl (KPO_4 buffer) was applied.

Column chromatography of *C. sorokiniana* extracts

A DEAE-cellulose column (20 ml) was prepared, equilibrated in Pipes buffer (above), and about 29 mg of protein from *Chlorella* were applied. The column was washed with about 40 ml of the same buffer before a 35 m*M* to 500 m*M* KCl gradient was added. Finally, 1.0 *M* KCl/Pipes buffer was added to remove residual protein from the column. Appropriate fractions with DQT and SDH activities in the gradient were pooled and dialyzed against 5 m*M* KPO_4 buffer, 20% glycerol, and 1 m*M* DTT at pH 7.2. An HA column (10 ml) was prepared with the 5 m*M* KPO_4 buffer, pH 7.2. A 15-ml sample from above was applied to the HA column and activities for DQT and SDH eluted in the wash fractions.

Celite 545 (shallow-gradient application of Fig. 5-1)

A 200-g amount of ground, frozen 7-day suspension cells was suspended in 200 ml of KPO_4 buffer, 7.2 pH, containing protectants and centrifuged as described above. A 3-ml

amount was removed for dialysis in Epps-KOH buffer and used as a crude extract. Ammonium sulfate was added to give 75% of saturation. From the slurry, 70 ml were removed and prepared for use as a concentrated crude extract. To the remaining slurry, 40 g of Celite 545 was added, stirred, poured into a column (90 ml vol) and the eluate collected into a cylinder. The column was washed with 65% $(NH_4)_2SO_4$ of saturation in Epps-KOH buffer (about 180 ml) and 8-ml volumes were collected. Buffer with $(NH_4)_2SO_4$ at 55% of saturation (120 ml) was added, followed by a shallow gradient of 600 ml from 55% to 45% of $(NH_4)_2SO_4$ saturation in Epps-KOH buffer. A step gradient of 180 ml of buffer at 45% of $(NH_4)_2SO_4$ saturation was applied before the second gradient of 45% to 0 of $(NH_4)_2SO_4$ saturation (400 ml) was added. A final wash of 120 ml of Epps-KOH buffer completed the elution schedule.

Gel filtration

A Sephadex G-200 gel filtration column was used to determine the native molecular weight of the S-proteins. The column was equilibrated with 50 m*M* Epps buffer, pH 8.6 and DTT. Three ml of concentrated crude extract, and concentrated SP-I or SP-II combined with 0.1 *M* sucrose were applied individually to the same column (column vol of 471 ml, void vol of 119.4 ml). Protein standards of 5 mg each,

alcohol dehydrogenase (150,000 D), BSA (66,000 D) and carbonic anhydrase (29,000 D) plus sucrose were applied to the column.

Purification of pathway proteins

Purification of the S-proteins

Crude extract of 540 ml was brought to 60% of ammonium sulfate saturation at 4°C while stirring and was used for the purification of the S-proteins.

Celite 545 chromatography. After stirring the 60% of ammonium sulfate saturation slurry for 30 min, 70 g of Celite 545 was added, with continued stirring for about 20 min. The slurry containing 605 mg protein was applied to a column and the eluate collected into a large cylinder. The column volume of 174 ml was washed with 60% of $(NH_4)_2SO_4$ saturation-Epps-KOH buffer containing 20% glycerol and 1.0 m*M* DTT at pH 8.6 and was collected in a separate cylinder. No activity was found in these eluates, although the protein level was very high. A 500-ml gradient from 60 to 40% of $(NH_4)_2SO_4$ saturation in the same buffer was applied and 8-ml fractions were collected. Next a 40% of $(NH_4)_2SO_4$ saturation-Epps-KOH buffer of about 130 ml was applied. A second 400 ml gradient of 40 to 0% of $(NH_4)_2SO_4$ saturation-Epps-KOH buffer was applied, followed by a wash of buffer without $(NH_4)_2SO_4$. Two peaks of SDH/DQT activities were located, pooled individually, and dialyzed against the Epps

buffer as above. Samples of each were saved for assay, and the remainder of each pool was used in the second purification step (643 ml, 206 mg of SP-I protein and 348 ml, 97 mg of SP-II protein).

CM-cellulose chromatography. CM cation exchanger columns were equilibrated with 50 m*M* Epps-KOH, 20% glycerol and 1 m*M* DTT at pH 8.6. SP-I was applied to a bed volume of 123 ml and SP-II was applied to a bed volume of 80 ml. Both SP-I and SP-II eluted in wash fractions and a small portion was saved for assay. The remaining portions of the preparations were used in the third step of purification.

DEAE-cellulose-52 chromatography. Two DEAE-52 anion exchanger columns were prepared and equilibrated in the same Epps-KOH buffer as the preceding step. Proteins were applied, SP-I to bed volume of 107 ml and SP-II to a bed volume of 72 ml, and washed with the same buffer. A 400-ml (SP-I) or 300-ml (SP-II) gradient from 0 to 0.6 m*M* KCl was applied to the columns. Fractions of about 6.5 ml were collected. Single peaks of activity, each eluting at about 0.3 m*M* KCL were individually pooled and dialyzed against 5 m*M* KPO_4 buffer containing 20% glycerol and 1 m*M* DTT at pH 7.3. Samples were saved for assay, and the remainder of each protein was used in the fourth step of purification.

Hydroxylapatite column chromatography. SP-I and SP-II were applied to HA columns equilibrated in the KPO_4 buffer of the previous step (100 ml and 50 ml bed volumes,

respectively). Each protein eluted in the wash and was applied to the fifth step of purification after samples were removed for assay.

DEAE KPO_4 chromatography. DEAE columns were equilibrated in 50 m*M* KPO_4 buffer at pH 7.3, before applying the proteins from the previous step. Gradients up to 0.3 *M* KCl were applied to each column. Single peaks of each protein were eluted, pooled and dialyzed into 50 m*M* Epps-KOH containing 20% glycerol and 1 m*M* DTT at pH 8.6. Samples were saved, and the remaining proteins were subjected to the sixth step of purification.

2'5'ADP Sepharose-4B affinity chromatography. An $NADP^+$-specific 2'5'ADP Sepharose-4B affinity column was prepared and equilibrated with the Epps-KOH buffer. The SP-I sample was applied to the column and washed with starting buffer before a 300-ml gradient from 0 to 0.18 *M* NaCl and 0 to 12 @8,25 μM $NADP^+$ was applied. The protein eluted at about 4 μM $NADP^+$ in the gradient. The SP-I Activity peak was pooled and dialyzed into the Epps-KOH buffer. A sample of SP-I was saved, and the remaining fraction was used in the seventh step of purification. The SP-II was applied to the affinity column similarly. Purification of SP-II was completed with this step at a final volume of 15 ml and total protein of 6 μg.

Final steps of SP-I purification. SP-I was applied once again to a DEAE column equilibrated in Epps-KOH buffer as previously described. A 200-ml gradient from 0 to 0.6 *M* KCl was applied. The SP-I activity peak fractions were pooled and dialyzed against Epps buffer. An aliquot was saved, and 83 ml containing 0.162 mg protein was applied to the affinity column for the final step of purification. The completed purification yielded 0.07 mg SP-I protein in 54 ml.

Purification of arogenate dehydrogenase

ADH was purified by two methods: A) by a series of steps of an established procedure which began with a 0 to 45% ammonium sulfate fractionation and ended with the $NADP^+$ affinity column (8), or B) by the elution of ADH from a Celite 545 column used to separate S-protein activities, followed by HA column chromatography, DEAE column chromatography, and the $NADP^+$ affinity column chromatography described above.

Purification of prephenate aminotransferase

PAT was purified by following an established procedure (7), which began with a 45 to 75% of ammonium sulfate saturation precipitation step, followed by a 70°C treatment to inactivate and then precipitate (by centrifugation) contaminating aminotransferases, and ended with a specific aminotransferase pyridoxamine phosphate (PMP) affinity column.

PAGE

SDS gel electrophoresis

SDS gel electrophoresis was prepared by following the procedure of Laemmli (51), with a 15% running gel and a 4% stacking gel. A running buffer of Tris/glycine/SDS at pH 8.8 was used and the gel was run at constant voltage of 63 V for about 8 h. The gel was stained for about 30 min in Coomassie Brilliant Blue R250 dye, destained and viewed.

Silver staining

Silver staining of an SDS gel as described by Pharmacia (Piscataway, NJ) was followed. Washes of methanol/acetic acid and ethanol/acetic acid were included before incubation of the gel in oxidizer, followed by addition of the silver stain, then developer, and finally an acetic acid/water wash to stop the reaction.

Native gel electrophoresis

A native PAGE was run according to the method of Shaw and Prasad (74). Gel concentrations were 7% for the stacking gel and 15% for the running gel. The running gel was pre-electrophoresed to remove the ammonium persulfate, and fluorescent light was used to solidify the stacking gel which contained riboflavin. The gel was run at constant voltage of 60 V for about 8 h.

Shikimate dehydrogenase activity stained gel

Optimal conditions for SDH activity stain on native PAGE included 20 mg $NADP^+$, 50 mg shikimate, 10 mg 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; (thiazolyl blue), and 2 mg N-methyldibenzopyrazine methyl sulfate (phenazine metasulfate) in 50 ml of 100 m*M* glycine buffer (without glycerol) at pH 9. Gels were incubated at 37°C for 10 to 20 min or at room temperature for 30 min to an hour. These conditions were used unless otherwise stated and were modified from the method presented by Davis (24).

Kinetic values

Shikimate dehydrogenase

Enzyme activities were assayed fluorometrically as described for SP-I and SP-II. In the forward physiological direction, either DHS or NADPH was held constant at saturating levels while the remaining substrate concentration was varied to obtain saturation curves. In the reverse of physiological direction, SHK and $NADP^+$ were assayed similarly. Double reciprocal plots of the substrate saturation data were used to determine the Km for each substrate.

Dehydroquinase

DQT activities were assayed spectrophotometrically as described for SP-I and SP-II at varying concentrations of DHQ to obtain saturation data, and then analyzed appropriately as above to determine the Km for each protein.

Inhibition studies of S-proteins

Enzyme was incubated for 10 min with p-chloromercuribenzoate or with possible protectants {DTT, cysteine or β-mercaptoethanol (βME)}. SDH was assayed at saturating conditions and DQT was assayed at 0.1 m*M* DHQ. For prevention experiments, DTT, cysteine, or βME was added about 10 min before addition of substrates and PCMB, and then rates were determined. For reversal experiments, PCMB was added to enzyme and buffer for 10 min before assay with substrates. After a rate (or no rate) was established, DTT was added to the reaction mix. Concentrations are as stated in Results.

Antibodies

Preparation of specific antibodies

Antibody preparations were produced by Kel-Farms (Alachua, FL) with repeated injections of purified SP-I over the course of several months until antibody titers, tested by Ouchterlony double diffusion method against SDH, ADH or PAT, were high enough to bleed the rabbits.

Purification of specific antibodies

Antibodies were purified following a BIO-RAD (Rockville Center, NY) procedure, by passing each over Econo-Pac 10DG columns for desalting and then passing through DEAE Affi-Gel Blue Econo-Pac 10DG columns for serum IgG purification. *E. coli* strain DH5α lysate was prepared and incubated with the

purified IgG. This step removed non-specific precipitant bands observed in the double-diffusion experiments. Pre-immune rabbit serum were purified similarly.

Antigen:antibody assay

Antigens (SP-I, ADH or PAT) from crude extracts or extracts from various purification steps were combined 1:1 (v:v) (or as stated) with antibodies. Samples were incubated at 37°C for 10 min and returned to an ice bath, then centrifuged (5 to 10 min) at full speed in a microfuge to precipitate the complexed antigen:antibody. The supernatants were assayed for enzyme activities. Controls were always performed with preimmune rabbit serum.

Ouchterlony assay

Ouchterlony plates were prepared with 1.5% agarose in sodium barbital and sodium azide and wells were prepared at precisely measured distances (66). Antibodies or antigens were diluted with 0.9% NaCl whenever necessary and then added to the wells. Plates were incubated at 37°C overnight.

Western blotting

Western blotting procedures from Sambrook et al. (68) were followed, and bands from either SDS or native PAGE were transferred to nitrocellulose filter paper. Membranes were incubated in blocking solution alone and in blocking solution containing diluted SP-I antibody for one h, washed, incubated further with blocking buffer containing alkaline

phosphatase-linked goat-anti-rabbit antibody for 1 h, and then incubated in STP buffer containing $MgCl_2$ and substrates, nitroblue tetrazolium and the p-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate. After 1 h, membranes were viewed and photographed.

Antibody precipitation of DQT and SDH from *C. sorokiniana*

Antibody made to SP-I was incubated 1:1, 2:1, or 4:1 with *Chlorella* extract (controls included preimmune antiserum and extract diluted with buffer). After 10 min at 37°C, and centrifugation for 5 min at full speed in a microfuge, preparations were assayed under standard conditions for SDH and DQT activities.

cDNA cloning and sequencing

Tobacco leaf cDNA library

A cDNA library prepared from *Nicotiana tabacum* var. SR1 tissue culture cells, inserted into pBluescript SK- plasmids and packaged into Lambda ZAP II vectors, was purchased from Stratagene, La Jolla, CA. *E. coli* XL1-Blue (a host strain used for immunological screening of libraries constructed in Lambda ZAP expression vectors) and *E. coli* SOLR (a non-suppressing host strain for use after excision of the pBluescript plasmid from the Lambda Zap vector) were included. Helper phage, ExAssist, efficient for *in vivo* excision of the plasmid from the Lambda Zap vector without replication of the phage genome was also included.

Preparation of cDNA libraries for screening

The cDNA library was first titered, then mixed with the freshly prepared host strain, XL1-Blue, plated with top agar on about 80 LB plates at about 20,000 PFU (about 1.6 X 10^6 PFU/ desired clone) and incubated at 37°C. Isopropylthio-*β*-*D*-galactoside treated nitrocellulose filters were applied about 6 to 8 h later and incubation was continued overnight. Filters were removed and the immunological screening procedure of Sambrook et al. (68) was followed.

Screening the cDNA library of Lambda ZAP II

Nitrocellulose filters removed from the plates were washed, soaked in blocking buffer containing dry milk, rocked gently in primary S-protein, ADH or PAT specific antibody/blocking buffer and washed several times. The filters were next exposed to secondary antibody, goat anti-rabbit antiserum, washed as before and incubated with substrates, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate and with 5 m*M* $MgCl_2$. The filters were kept in the dark and gently rocked for one to two hours. Plaques corresponding to the small, deep blue spots on the filters were cored from plates, dispensed into SM buffer plus chloroform and stored at 4°C for titering and plaque purification.

Excision of Pbluescript from the Lambda ZAP II vector

Purified phage stocks were incubated with *E. coli* strain XL1-Blue and ExAssist helper phage to excise the pBluescript plasmid from the Lambda ZAP II vector for 2 to 2.5 h at 37^{0}C in 2XYT media (see Stratagene instruction manual for details), heated to 70^{0}C and centrifuged. The supernatant, containing the plasmid, was incubated with host *E. coli* strain SOLR, spread on LB plates containing 50 mg ml^{-1} ampicillin and incubated overnight at 37^{0}C. Colonies from these plates were streaked on fresh LB-AMP plates for immediate use and were also stored in DMSO for future use.

DNA purification

Plasmid DNA was purified by two methods. Method 1: The pBluescript plasmids containing putative cDNA clones coding for the S-protein, ADH or PAT were purified for sequencing by the method of the DNA Sequencing Core Facility, ICBR, University of FL. A colony from the *E. coli* SOLR strain from the LB-AMP plate above was incubated overnight in 10 ml of LB-AMP medium in a 50 ml tube for each clone sample. The cultures were centrifuged and the pellets were resuspended in GTE (68) buffer. Treatment with NaOH/SDS was followed by neutralization with potassium acetate at pH 4.0, and centrifugation was carried out to remove cellular debris. The supernatants were treated with DNase-free RNase and extracted twice with chloroform. DNA was precipitated with isopropanol and washed with 70% ethanol. To precipitate the

plasmid DNA, the pellets were first dissolved in sterile water, then 4*M* NaCl and 13% PEG_{8000} was added, followed by incubation on ice before centrifugation at 4°C. The pellets were rinsed in ethanol, dried under vacuum and resuspended in sterile water. DNA concentrations were determined spectrophotometrically at 260 nm and by agarose gel electrophoresis with a known DNA standard. Method 2: The 10 ml cultures, as prepared above after centrifugation, were treated with prepared solutions provided by Promega (Madison, WI) in the Magic Miniprep DNA purification kit. The DNA was passed through a mini-column and dissolved in GTE (Promega instructions for preparation) buffer. This rapid method is preferred for sequencing on a Li-Cor sequencer available in the Department of Microbiology and Cell Science.

Competent cells of *E. coli* strains

E. coli strains DH5α, AB1360 (*aroD*) and SK494 (*aroE*) were grown overnight in 50-ml tubes and inoculated into 1-L flasks containing 200 ml LB medium. The cells were incubated at 37°C in a shaker at 300 rpm for several hours until a spectrophotometric reading at $A_{600\ nm}$ between 0.4 and 0.5 was reached. Cells were harvested by centrifugation in sterile tubes after incubation on ice, treated with 100 m*M* $CaCl_2$ as described by Sambrook (68), resuspended into 100 m*M* $CaCl_2$ with 15% glycerol, aliquoted into microcentrifuge tubes, dropped into liquid nitrogen and stored at -70°C

until use. Controls were prepared by streaking auxotrophic mutant strains on M9 medium plates +/- aromatic amino acids.

Transformation of plasmid DNA

Either pUC18, pGEM5zf(+), or pBluescript SK+ plasmids were incubated with competent cells of DH5α on ice, heated to 42°C for 90 sec, cooled in an ice bath before addition of SOC medium (68). The cells were then incubated at 37°C for 1 h before spreading on LB-AMP plates. A colony from these transformed cells was streaked onto a fresh LB-AMP plate. Controls included spreading of competent cells without plasmid or plasmid without insert onto LB-AMP plates.

Functional complementation

E. coli mutant strains, *aroD* (DQT^-) and *aroE* (SDH^-), were prepared for transformation with the pBluescript plasmids carrying cDNA clones and were plated on LB-AMP plates. After growth, colonies were streaked onto M9 plates. Controls included nontransformed competent cells or plasmid spread on LB-AMP plates, and competent mutant strains spread on M9 plates +/- aromatic amino acids.

Initial sequencing of cloned cDNA

Purified pBluescript plasmid with cloned inserts were submitted to the DNA Sequencing Core Facility, ICBR, UF, for initial 5′ and 3′ end sequencing (from the T3 and T7 promoter sites).

Subcloning cDNA for complete sequence analysis

Subcloning of cDNA inserted into pBluescript was performed by use of appropriate restriction enzymes to obtain fragments for sequencing both strands of the entire cDNA. Plasmids used for fragment insertion {pUC18, pGEM5zf(+), or pBluescript SK+} were incubated with the same restriction enzyme(s), and were treated with calf intestinal alkaline phosphatase (CIAP) (when only one RE was used) to prevent rejoining of ends during ligation. Cloned cDNA fragment samples and plasmid samples were loaded onto 1% DNA agarose gels at 70V for about 1 h. Fragments and linear plasmid DNA were excised and treated with the Gene Clean system (Bio 101, LaJolla, CA), prior to ligation and transformation.

Li-Cor sequencing of subcloned cDNA fragments

Plasmid DNA was denatured and reacted with forward or reverse fluorescent primers, enzymes, 7-Deaza dGTP sequence extending mix and NTPS according to the directions supplied by Li-Cor (Lincoln, NE). Three μl of each of four prepared sample mixtures containing one of the four dideoxy nucleotides was loaded onto wells of a urea polyacrylamide gel set into the Li-Cor model 4000L sequencer. Parameters were set in a computerized data collection file and the sequencer was run overnight. The sequences were analyzed in a data analysis computerized system.

N-terminal sequencing

About 1 pmol of purified SP-I protein was prepared for N-terminal sequencing on SDS PAGE, followed by transfer of the protein onto 3MM paper by electrophoresis. Mini-blot system protocol and amino acid analysis by acid hydrolysis was provided by the Protein Chemistry Core Facility, Interdisciplinary Center of Biotechnology Research, University of Florida.

Computer analysis

The GCG computer software package (26) was used to analyze nucleotide and deduced amino acid sequences of cloned cDNAs.

Biochemicals

Arogenate (90% pure) was isolated from a multiple auxotroph of *Neurospora crassa* ATCC 36373 (42) or isolated from a tyrosine auxotroph of *Salmonella typhimurium* (4). Prephenate (85% pure) was prepared as the barium salt from culture supernatants of the tyrosine auxotroph of *S. typhimurium* and was converted to the potassium salt with excess K_2SO_4 prior to use. Dehydroquinate (90% pure) was chemically synthesized by following the method of Haslam et al. (37) using a platinum-catalyzed dehydrogenation of quinic acid (about 90% pure). 2'5' ADP Sepharose-4B and AH-Sepharose 4B were purchased from Pharmacia, Piscataway, NJ. Bradford reagent was purchased from Bio-Rad, Rockville

Center, NY. DTT and hydroxylapatite were purchased from Research Organics, Inc. Cleveland, OH. Shikimate, NADP, NADPH, benzamidine, leupeptin, and PMSF were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were purchased through Fisher Scientific Co., Orlando, FL.

CHAPTER III

PHYSIOLOGICAL PROFILE OF AROMATIC PATHWAY ENZYMES IN PLANTS

The activities of dehydroquinase and shikimate dehydrogenase (of the common portion of aromatic biosynthesis), and prephenate aminotransferase, arogenate dehydratase and arogenate dehydrogenase (of the post-prephenate portion of the pathway) were examined under selected physiological conditions in the higher plant *N. silvestris* and in the green alga *Chlorella sorokiniana*. Prephenate aminotransferase from *Chlorella* exhibited several of the striking characteristics of higher plant enzyme.

Arogenate dehydratase activities were followed in a growth culture cycle of *N. silvestris* suspension cells similarly as was followed for PAT and ADH (12).

Results

Specific activities from crude extracts

Specific activities have been compared for several aromatic amino acid pathway enzymes of *N. silvestris* cultured cells and from the green alga *C. sorokiniana* (Table 3-1). A constant ratio of about 5 for SDH and DQT activity was found in *N. silvestris*, compared to a ratio of 1 for crude extracts of *C. sorokiniana*. The activities of DQT and

SDH from *Chlorella* extract were unaffected when incubated with SP-I *N. silvestris* specific antibody. Activity was not found when QDH was assayed in *N. silvestris* and *C. sorokiniana* crude extracts.

Table 3-1. Specific activities from *N. silvestris* and *C. sorokiniana* aromatic pathway enzymes.

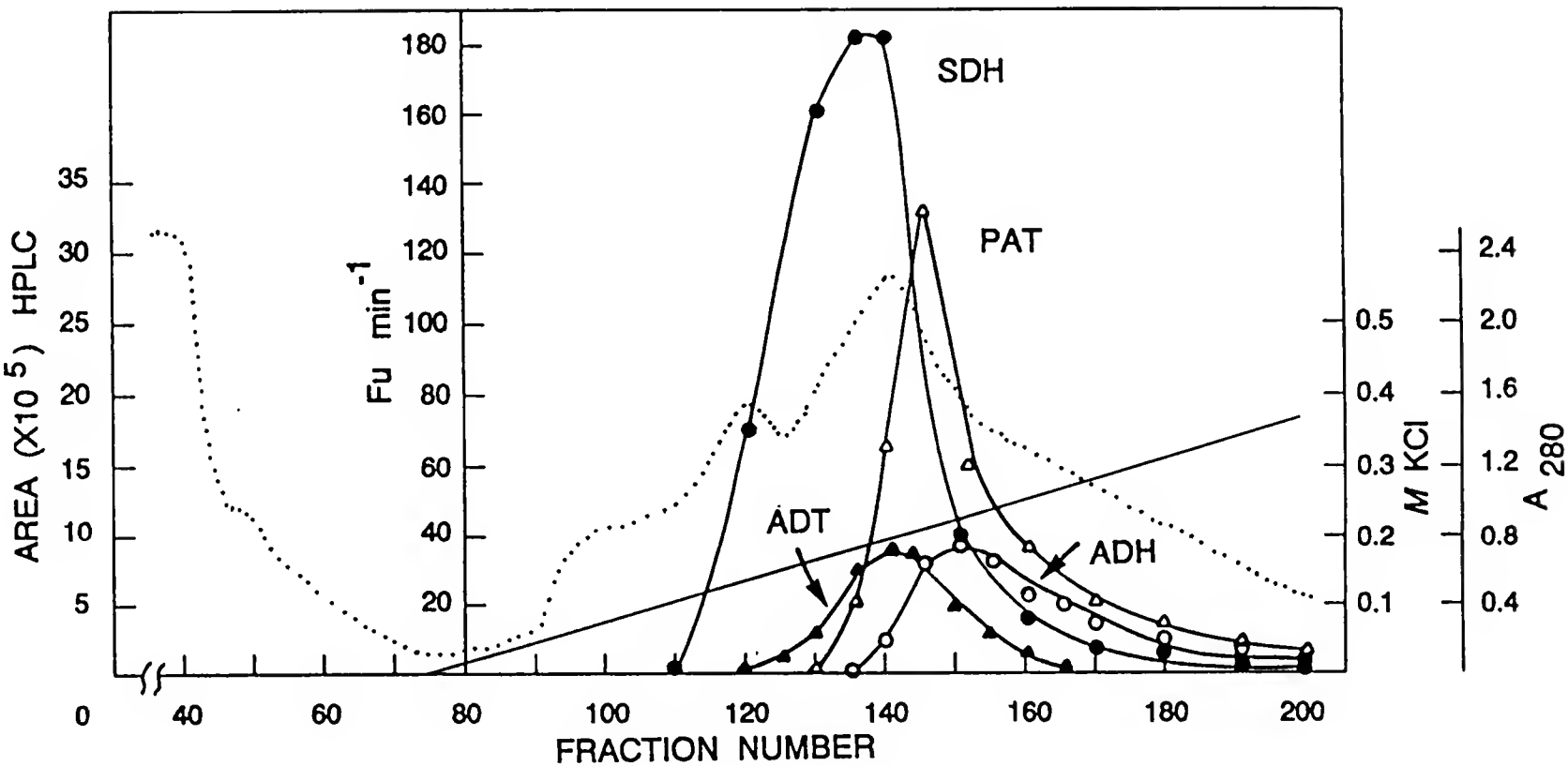
	Specific Activity (nmol min^{-1} mg^{-1} protein)	
Enzyme	*N. silvestris*	*C. sorokiniana*
QDT	12.00	6.20
SDH	60.00	6.00
PAT	16.00	3.75
ADH/NADP	1.00	NF
ADH/NAD	NF	NF
PDH/NADP	NF	NF
PDH/NAD	NF	NF
ADT	0.6	NF
PDT	NF	NF

NF, not found; Crude extracts were used for assay.

Separation of activities

Column chromatography was used to separate the enzymes of interest for this study from *N. silvestris* and *C. sorokiniana*. DQT and SDH of the common pathway were not separable in *N. silvestris* or in *C. sorokiniana* after two steps of column chromatography (DE-52 and HA). Figure 3-1 shows a DEAE-cellulose chromatographic profile of *N. silvestris* suspension cell extract with overlapping

Fig. 3-1. DEAE-cellulose chromatography of aromatic pathway enzymes from *N. silvestris*. Extract of five day suspension cells was applied to a DE-52 column as described in Materials and Methods. PAT (△), ADH (O), ADT (▲), and SDH (●) eluted in the KCl gradient (—) and protein was monitored (…).

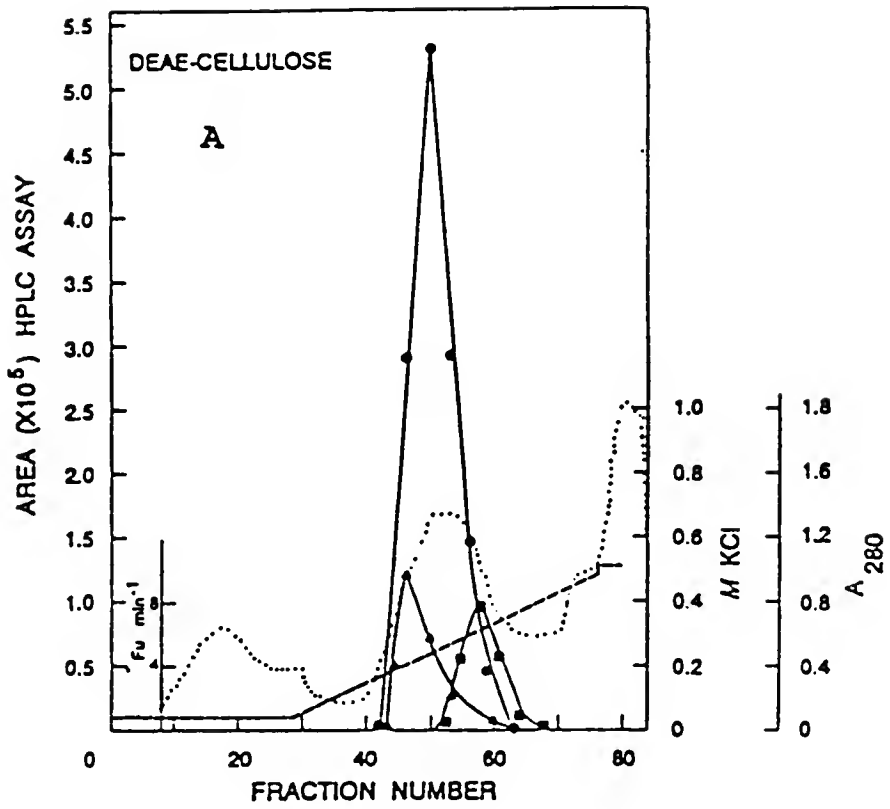

AREA (X10 5) HPLC
Fu min-1
M KCl
A 280
SDH
PAT
ADT
ADH
FRACTION NUMBER
0
40
60
80
100
120
140
160
180
200
5
10
15
20
25
30
35
20
40
60
80
100
120
140
160
180
0.1
0.2
0.3
0.4
0.5
0.4
0.8
1.2
1.6
2.0
2.4

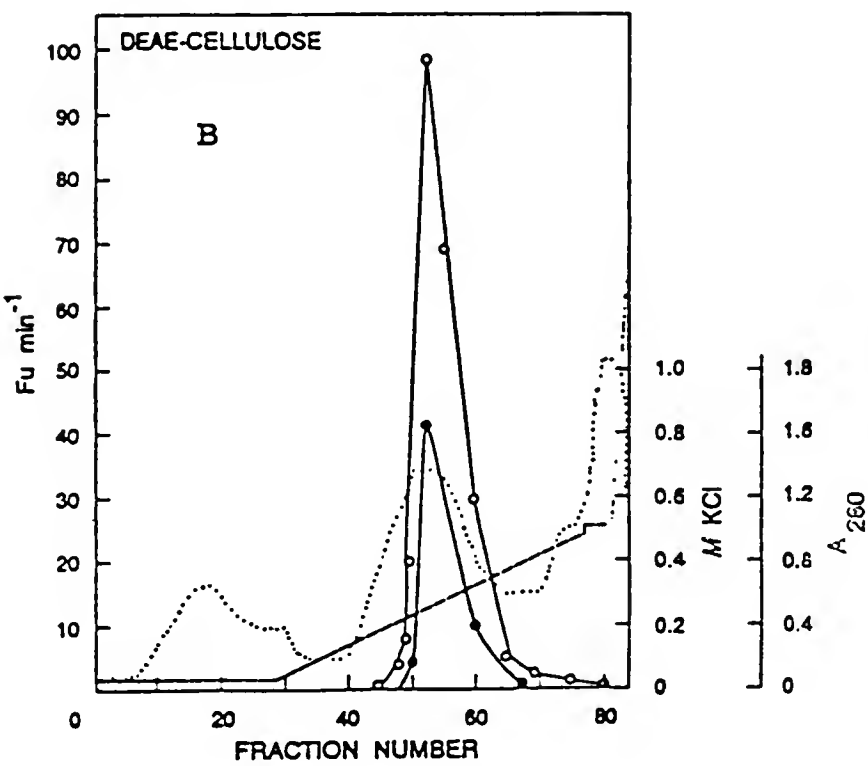
enzyme activities of PAT, ADH, ADT and SDH (DQT activity was coincident with SDH activity; not shown). Although ADT and ADH activities were not found in crude extracts of *C. sorokiniana*, they were located in DEAE-cellulose fractions at very low activities as shown in Fig. 3-2A. ADH was specific for cofactor $NADP^+$. Prephenate dehydratase and prephenate dehydrogenase activities were not found in these column fractions. The ADH and ADT activities overlapped those of PAT, DQT, and SDH in the KCl gradient fractions. The coeluting DQT and SDH activity peak fractions, as shown in Fig. 3-2B, were pooled, dialyzed and applied to an HA column where they once again coeluted, this time in the wash fraction, data not shown.

PAT in *C. sorokiniana*

Several of the unique properties earlier shown for prephenate aminotransferase in *N. silvestris* suspension cells (5) were studied in *C. sorokiniana* extracts. A high temperature optimum of about 70^0C for PAT was also found in the alga extract as shown in Fig. 3-3. In Table 3-2, it is shown that PAT from *Chlorella* has a preference for GLU as the amino acid donor in combination with the keto acid substrate, PPA. As found in higher plants, ASP was utilized at about 50% of the activity with GLU. Slight activity was seen with TYR as the amino acid donor in *C. sorokiniana* but not in *N. silvestris*.

Fig. 3-2. DEAE-cellulose chromatography of aromatic pathway enzymes from *C. sorokiniana*. Alga extract was applied to a DE-52 column as described in Materials and Methods. A) PAT (●), ADH (■), and ADT (▲) eluted in the KCl gradient (—) and protein was monitored (…). B) SDH (O) and DQT (●) eluted in the gradient.


DEAE-CELLULOSE
A
AREA (X10^5) HPLC ASSAY
$f_{Fu\ min^{-1}}$
M KCl
A_{280}
FRACTION NUMBER


DEAE-CELLULOSE
B
Fu min^{-1}
M KCl
A_{280}
FRACTION NUMBER

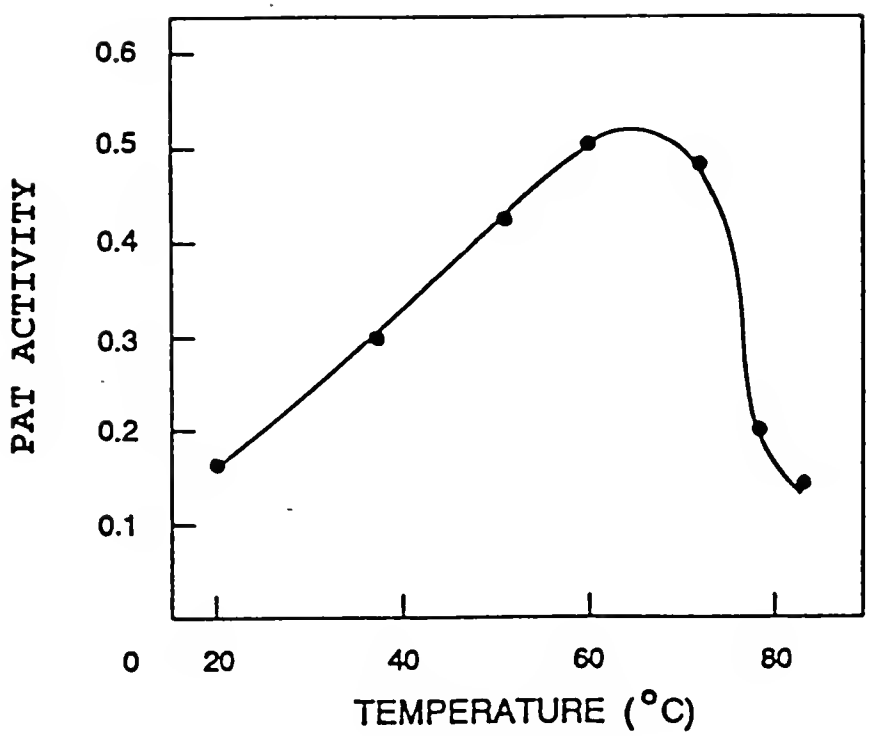


Fig. 3-3. Temperature optimum for activity of prephenate aminotransferase in *C. sorokiniana*. The temperatures range used was from 20°C to 80°C for 5 min.

Table 3-2. Prephenate aminotransferase amino acid donor specificity in *C. sorokiniana*.

L-Amino Acid Donor (10 m*M*)	Specific Activity
GLU	16.0
ASP	8.0
TYR	2.0
LEU	0
ALA	0

[a]A concentration of 1 m*M* PPA was used in combination with the amino acids. S. A. is nmol min^{-} mg $protein^{-}$

When *C sorokiniana* extract was incubated at 65°C for 10 min and then assayed at 37°C, activity with PPY as keto acid substrate was lost, but activity with PPA remained constant. At 70°C for 20 min, however, about 60% of the PAT activity was lost (Table 3-3).

Table 3-3. Prephenate aminotransferase activity after thermal treatment in *C. sorokiniana*[a].

Aminotransferase Couple	No treatment	65°C 10 min	70°C 10 min	70°C 20 min
PPA/GLU	0.61	0.60	0.61	0.25
PPY/GLU	0.29	0	0	0

[a]Reaction mixtures were incubated at 37°C following thermal treatment. Assays contained 1 m*M* PPA or 10 m*M* PPY, 10 m*M* GLU and 0.05 mg protein.

ADT activities in cultured *N. silvestris* cells.

When *N. silvestris* stationary phase cells were diluted into fresh medium, a substantial elevation of soluble protein occurred which increased until day 2, leveled off between days 2 and 3, and began a steady decline between days 3 and 7 as shown in Fig. 3-4B. This rise ceased before mid-exponential growth was reached. That soluble protein synthesis was terminated before reaching a maximum was indicated by the finding that EE cells achieved a two-fold higher content of soluble protein. EE cells exhibited a consistently higher soluble protein content of about 360 mg g dry wt.$^{-1}$.

During the transition of cell populations in lag phase to exponential-phase growth, new synthesis of macromolecular constituents must have occurred. Figure 3-5A shows that the rise in ADT activity expressed as units of activity per g dry wt., paralleled the rise in soluble protein between subculture and day 2 (Fig. 3-4B). The rise in ADT activity levels continued until day 3, whereas overall soluble protein content plateaued between days 2 and 3. Thus, specific activity or activity expressed on a cell basis peaked at day 3. Since the specific activity of E cells and EE cells are similar and since the ratio of soluble protein to dry weight is greater in EE cells than in E cells, the activity of ADT expressed as units per g dry wt was greater in EE cells than in E cells. The decline in specific

Fig. 3-4. Growth curve of *N. silvestris* in suspension cultures. A) Cell growth monitored as cell count or as dry weight. The physiological stages of growth: lag (L), exponential (E) and stationary (S) are indicated in each panel. B) Soluble protein content was determined in samples harvested daily and was related to the dry wt. and cell number. Comparable protein values obtained with EE cells (average of ten determinations) are indicated with stippled bars, the widths of which indicate the range of variation.

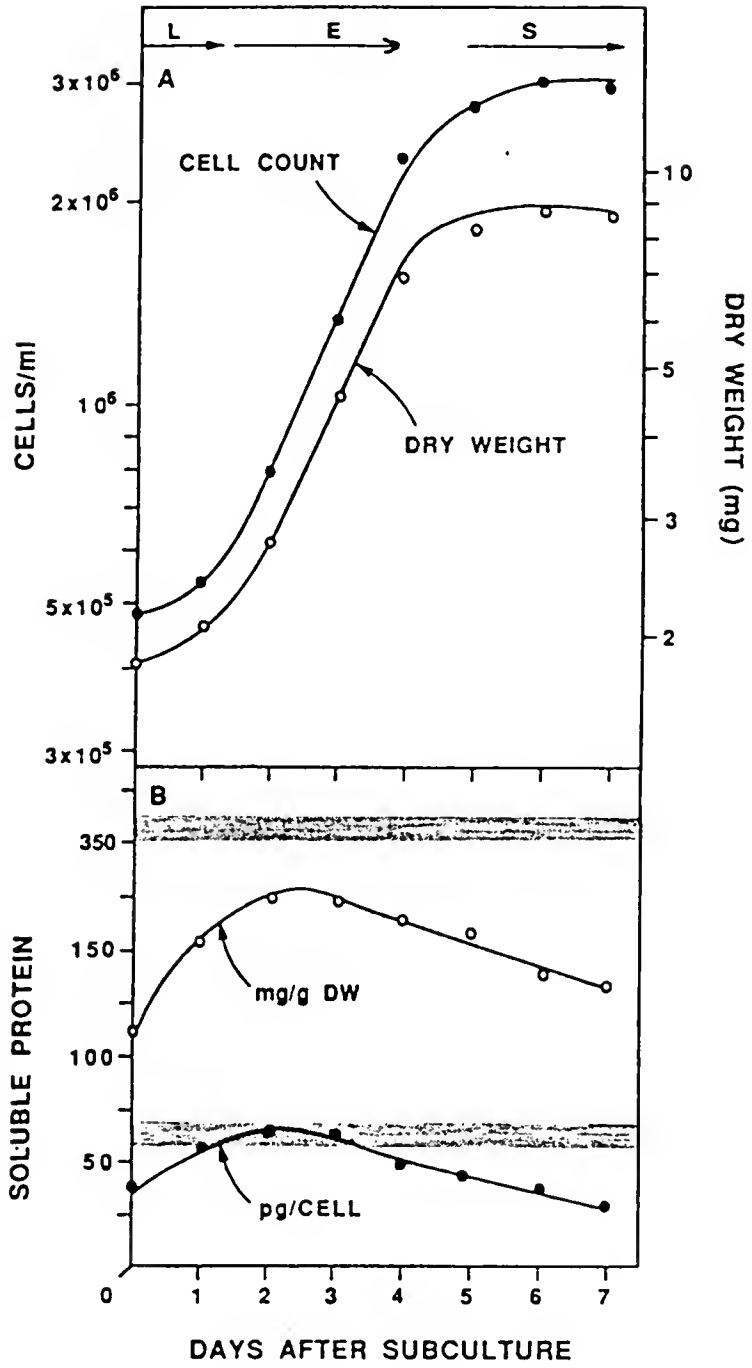
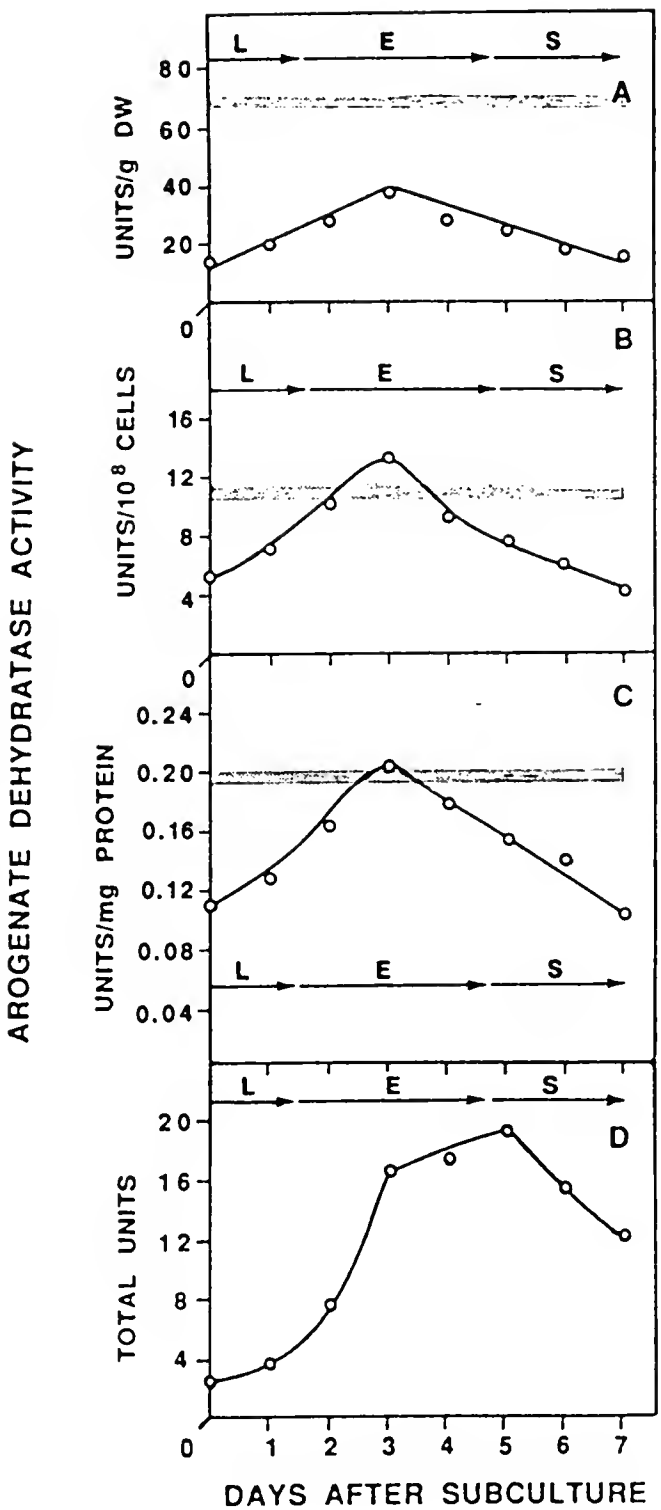

L
E
S
A
3x10⁶
2x10⁶
10⁶
5x10⁵
3x10⁵
CELLS/ml
CELL COUNT
DRY WEIGHT
10
5
3
2
DRY WEIGHT (mg)
B
350
150
100
50
SOLUBLE PROTEIN
mg/g DW
pg/CELL
0
1
2
3
4
5
6
7
DAYS AFTER SUBCULTURE

Fig. 3-5. Arogenate dehydratase activities followed throughout a growth curve of *N. silvestris*. Activity was followed in samples harvested daily throughout the growth curve shown in Fig. 2-4. A) Activities expressed as units/g dry wt., B) as units/cell, C) as specific activity. The stippled bars indicate the corresponding activities found in EE cells (average of 10 determinations), the width of the bars indicating the range of variation. D) total cumulative units of activity per culture. See Fig. 2-4 for meaning of L, E and S.


AROGENATE DEHYDRATASE ACTIVITY
L
E
S
A
UNITS/g DW
80
60
40
20
0
B
UNITS/10⁸ CELLS
16
12
8
4
0
C
UNITS/mg PROTEIN
0.24
0.20
0.16
0.12
0.08
0.04
D
TOTAL UNITS
20
16
12
8
4
0
1
2
3
4
5
6
7
DAYS AFTER SUBCULTURE

activity (Fig. 3-5C) and units of activity per cell (Fig. 3-5B) between days 3 and 5 (mid-to late-exponential growth reflected a rate of enzyme synthesis that was slower than the growth rate since total units of activity (Fig. 3-5D) continued to increase during that time. From days 5 to 7 the decline in specific activity reflected a net loss of enzyme activity.

Discussion

C. sorokiniana aromatic-pathway enzyme activities appear to be somewhat lower but consistent with those found in higher plants. The S-protein from *N. silvestris* must be antigenically different from that of *C. sorokiniana*, since the S-protein specific antibody did not precipitate the protein from *Chlorella*. Specific antibodies made to PAT and ADH are now available (see Chapter V) to test cross reactions with these proteins from *Chlorella*.

Although the coincident DQT and SDH activity peak from the DEAE column which eluted protein from *C. sorokiniana* extract was only purified through two steps, it is conceivable that further purification would yield a bifunctional S-protein like that found in higher plants. Activities from column fractions indicated that DQT activity was about half that seen for SDH, whereas crude extracts indicated that activities were about equal. Possible explanations would be that (i) more than one protein is

present in *Chlorella* for DQT and/or SDH, (ii) some component in crude extract inhibits the SDH activity or (iii) DHS, the product of the reaction is being utilized by another protein. The second possibility may also apply to ADT and ADH since activity was not found in crude extracts but was located in column fractions.

Most interesting, is the similarity of PAT in *Chlorella* to that of higher plants, each having the high optimal temperature for activity. At 65°C, the aminotransferase(s) using the PPY/GLU couple was inactivated. The high specificity of *Chlorella* for the substrate couple of PPA and GLU was also consistent with higher plant PAT (5, 6). From these data, it is suggested that *C. sorokiniana* has an aromatic biosynthetic pathway similar to that established for higher plant chloroplasts. Supportive evidence would include the isolation of *Chlorella* chloroplasts to determine the location of the aromatic pathway enzymes. The isolation and sequencing of cDNA clones coding for S-protein, PAT and ADH (see Chapter VI) may be useful as probes to clone the appropriate genes in *Chlorella*.

Arogenate dehydratase activities paralleled a similar experiment with respect to the chloroplast isoenzyme of DAHP synthase (Ds-Mn), where growth of cells and enzyme activities where followed (32). Both enzymes rise in exponential growth and decline in stationary phase of growth. ADT exhibited an opposite pattern of activity to

three other aromatic pathway enzymes, SDH, PAT, and ADH. The three enzymes are known to exist in the chloroplast of higher plants, but the extent to which a fraction of each of the total activities measured might be derived from the cytosol is unknown. All three enzymes exhibited elevated levels of activity in stationary phase and minimal levels of activity in exponential-phase growth (11). Aromatic-pathway enzymes may be sorting out into two different patterns of expression for reasons yet to be elucidated. Alternatively, since enrichment of Ds-Co (the cytosolic isoenzyme of DAHP synthase), occurred in stationary phase, rising levels of SDH, PAT and ADH in stationary phase may reflect the expression of cytosolic isoenzymes. If so, the rise in activity of cytosolic isoenzymes exceeds the decrease of the plastidial isoenzyme counterparts. These data suggest that while ADT is present in the chloroplast pathway, it may not function in the cytosol.If so, perhaps prephenate dehydratase of the alternative route (activity, not as yet determined in plants, see Fig. 1-1), functions in the cytosol to synthesize PHE that may be used extensively for secondary metabolism.

CHAPTER IV

PURIFICATION OF PROTEINS FROM THE AROMATIC AMINO ACID PATHWAY

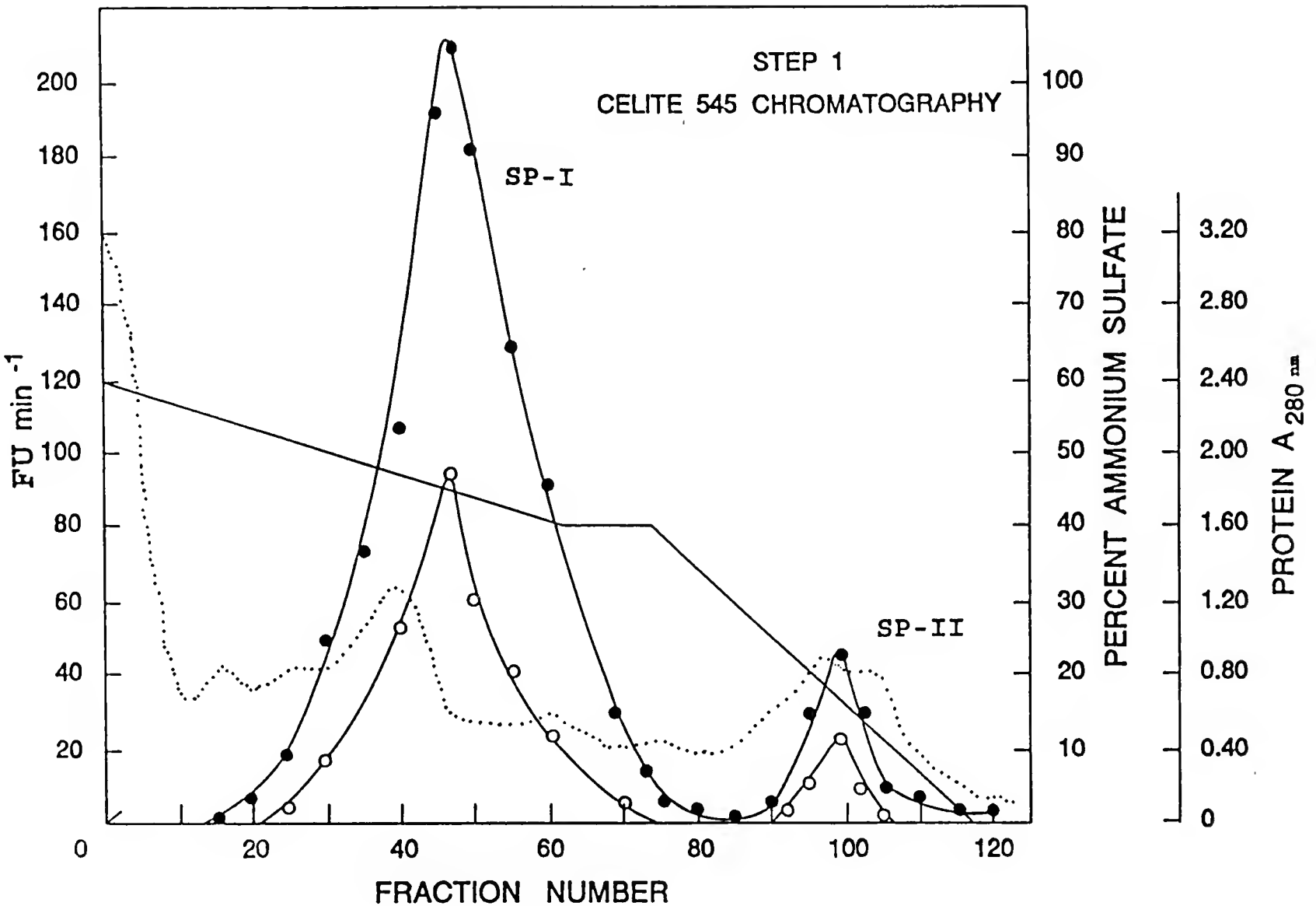
This chapter includes the separation and purification of the common pathway bifunctional DQT/SDH proteins from *Nicotiana silvestris* suspension cells. Six to eight steps of column chromatography, including a specific $NADP^+$ affinity column have been used. Prephenate aminotransferase and arogenate dehydrogenase were also purified by a series of chromatographic steps.

Results

DQT/SDH purification

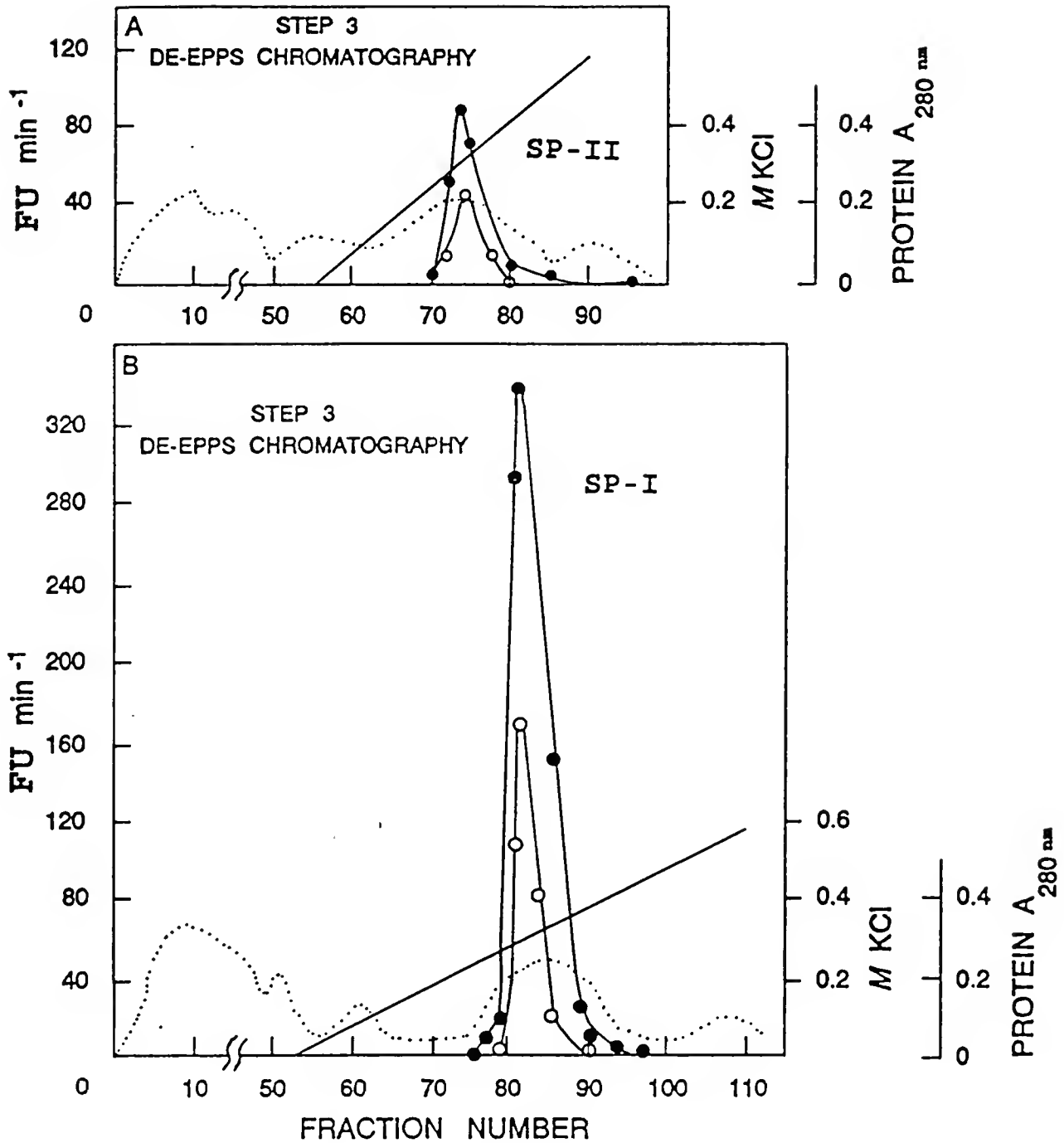
In crude extracts, activities for SDH and DQT (specific activity of 61 and 13, respectively) were detected by fluorometric or spectrophotometric assays. The enzyme(s) are not stable at 4^0C after several days of storage, unless glycerol and DTT are present. Under these conditions activities remained stable to freeze/thaw, at either -20 or -70^0C. Two peaks of activity for the bifunctional S-protein were eluted from a Celite 545 column with an ammonium sulfate gradient (Fig. 4-1). Elution of the major peak began at about 50% of ammonium sulfate saturation and was

Fig. 4-1. Elution profile of SP-I and SP-II from Celite 545 column chromatography. Two activities for SDH (●) were separated by a decreasing gradient of ammonium sulfate (solid line) on a Celite 545 column at about 50% and 25% saturation of ammonium sulfate as labeled. Dehydroquinate dehydratase (O) activity peaks were coincident with the SDH activity peaks. Protein was monitored at $A_{280\ nm}$ (…). See Materials and Methods for details of chromatography.


STEP 1
CELITE 545 CHROMATOGRAPHY
SP-I
SP-II
FU min -1
PERCENT AMMONIUM SULFATE
PROTEIN A 280 nm
FRACTION NUMBER
0
20
40
60
80
100
120
140
160
180
200
10
30
50
70
90
0.40
0.80
1.20
1.60
2.00
2.40
2.80
3.20

almost completely removed from the column by the end of a 45% of ammonium sulfate saturation step. The major activity peak fractions were completely separated from fractions containing the minor peak of activity and eluted at about 25% of ammonium sulfate saturation. Fractions with DQT activity were located coincidently with the two peak of SDH activity. There were no column fractions containing only one of these two activities. Fractions from each activity peak were pooled and labeled SP-I for the major peak of activity and SP-II for the minor peak of activity. Proteins (SP-I and SP-II) were further purified similarly by chromatography steps. It was calculated from this chromatographic step that the major peak, SP-I, accounts for 90% or greater of the total activity in the crude extract. The fold purifications calculated for the SP-I and SP-II were based on this assumption. The proteins eluted in the wash fractions of the CM columns in the second chromatographic step of purification. Figure 4-2 shows profiles of SP-I and SP-II elutions from the third step of purification on DEAE-cellulose columns in Epps buffer at pH 8.6. Each protein peak eluted at about 0.3*M* KCl in the gradient and had coincident DQT and SDH activities. The fourth chromatographic step was an HA column which eluted each protein in the wash fractions. This step was followed by another DEAE column, equilibrated at a lower pH of 7.2. Each bifunctional protein eluted in the gradient of an $NADP^+$

Fig. 4-2. Elution profiles of SP-I and SP-II from DEAE- cellulose chromatography. The third step of purification of the two S-proteins was a DEAE-cellulose chromatographic column equilibrated in EPPS buffer (see Materials and Methods for details). SDH (●) activity remained coincident with DQT (O) activity for both SP-II (panel A) and SP-I (panel B). The proteins began to elute at about 0.25*M* KCl of the gradient (solid line). Protein profiles were followed at $A_{280\ nm}$ (...).


A
STEP 3
DE-EPPS CHROMATOGRAPHY
SP-II
FU min -1
120
80
40
0
0.4
0.2
M KCl
0.4
0.2
0
PROTEIN A 280 nm
10
50
60
70
80
90
B
STEP 3
DE-EPPS CHROMATOGRAPHY
SP-I
FU min -1
320
280
240
200
160
120
80
40
0
0.6
0.4
0.2
M KCl
0.4
0.2
0
PROTEIN A 280 nm
10
50
60
70
80
90
100
110
FRACTION NUMBER

specific 2'5'-Sepharose-4B affinity column at 1 μM $NADP^+$ and 18 mM NaCl (Fig. 4-3). Activities for DQT and SDH remained coincident for each protein. The fold purification at this step was 195 for SP-I and 918 for SP-II. Only SP-I was purified further by repeating the DEAE-cellulose chromatography step (Epps-KOH buffer), followed by a second application on the affinity column. The final fold purification was 1077 for the SP-I. Purification yields, purities, specific activities and ratios of SDH/DQT activities are shown in Table 4-1 and in Table 4-2.

Throughout the purification of SP-I and SP-II, the ratio of shikimate dehydrogenase to dehydroquinase activity was calculated to be within the range of 4.7 to 6.2 for SP-I (at an average of about 5.1) and within a range of 4.4 to 5.4 for SP-II (at an average of about 4.8). Each protein was concentrated on a PM-10 membrane to about 1 or 2 ml before storage at -70^0 C.

Purification of ADH and PAT

When ADH was purified along with the SP-II, it was realized that they eluted with overlapping activity peaks on the Celite 545 column using the decreasing ammonium sulfate gradient at about 25 to 20% of saturation as shown in Fig. 4-4. A critical step of separation occurred when SDH did not bind to HA and ADH bound to HA (Fig. 4-5). Without the inclusion of this step, the two enzymes co-purified throughout the entire purification procedure, both eluting

Fig. 4-3. Elution profiles of S-proteins from an $NADP^+$ specific affinity column. A 2'5'-Sepharose-4B column with a combined $NADP^+$ (0-10 u*M*) and NaCl (0-0.16*M*) gradient was used as final steps of purification of the S-proteins, each eluting at about 0.018*M* NaCl and 1u*M* NADP. Both SDH (●) and DQT (O) activities coeluted. A) step six of purification of SP-II protein. B) step eight of purification of SP-I protein.

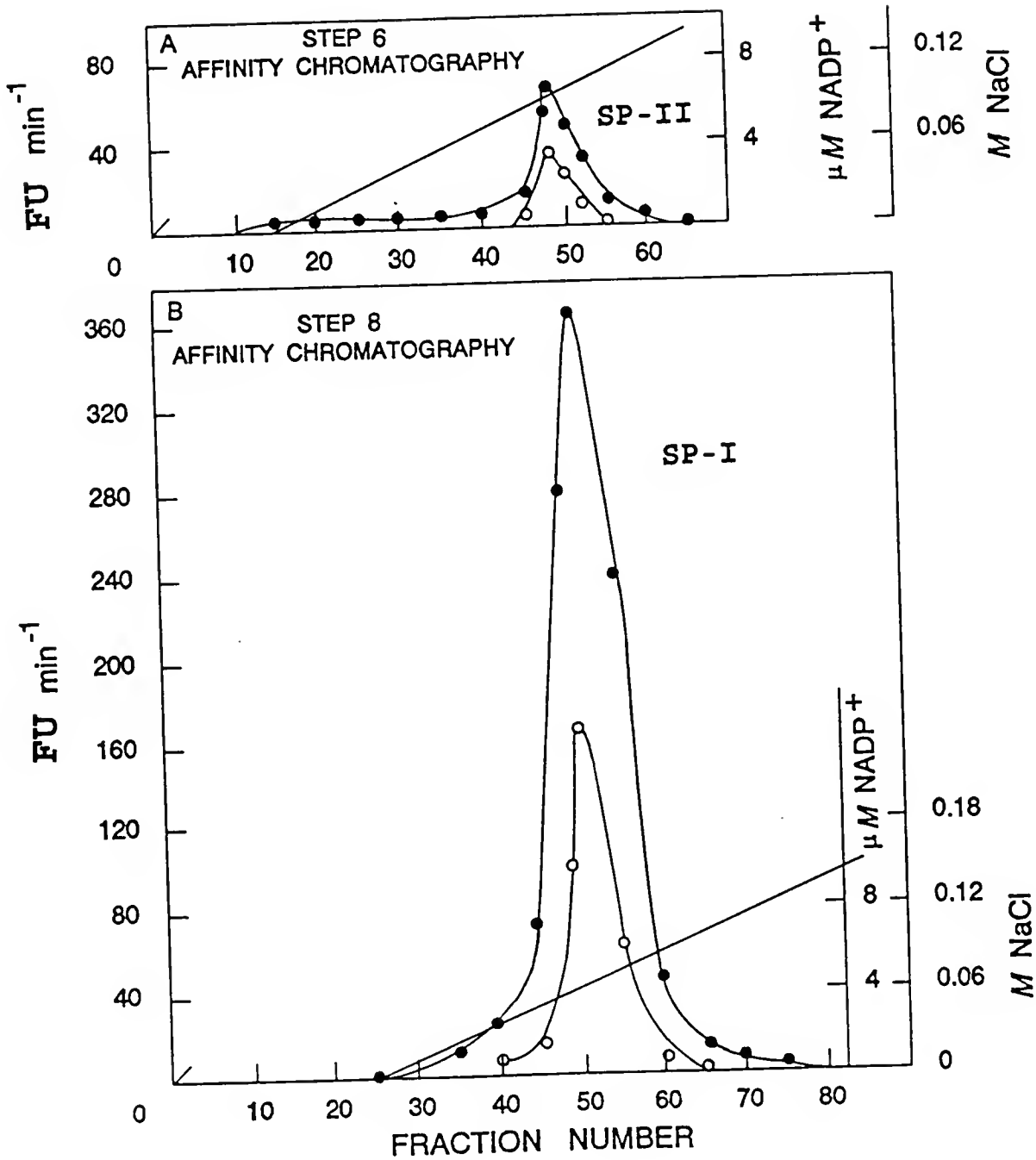

A
STEP 6
AFFINITY CHROMATOGRAPHY
SP-II
FU min-1
μM NADP+
M NaCl
B
STEP 8
AFFINITY CHROMATOGRAPHY
SP-I
FU min-1
μM NADP+
M NaCl
FRACTION NUMBER

Table 4-1. Purification of S1 protein from *N. silvestris* suspension cultured cells.

Step	Vol ml	Total Protein mg	SDH SA	SDH Purity fold	SDH Yield %	DQT SA	DQT Purity fold	DQT Yield %	Ratio S/D
Crude	540	605	56	1	100	12	1	100	4.7
Celite	643	206	126	2	70	25	2	65	5.0
CM-52	720	49	412	7	55	94	8	59	4.4
DEAE-1	279	35	561	10	53	113	9	50	5.0
HA	618	8.7	1,515	27	36	257	22	28	5.9
DEAE-2	106	1.95	4,609	82	24	941	78	23	4.9
Aff-1	88	0.42	14,500	259	17	2,342	195	13	6.2
DEAE-3	83	0.16	33,878	605	15	7,365	614	15	4.6
Aff-2	54	0.07	65,877	1176	9	12,923	1077	12	5.1

S/D is shikimate dehydrogenase/dehydroquinase.

Table 4-2. Purification of S2 protein from *N. silvestris* suspension cultured cells.

Step	Vol ml	Total Protein mg	SDH SA	SDH Purity fold	SDH Yield %	DQT SA	DQT Purity fold	DQT Yield %	Ratio S/D
Crude	540	605	5	1	100	1	1	100	4.6
Celite	348	97	26	5	68	5	5	64	5.0
CM-52	345	69	31	6	59	8	8	73	4.4
DEAE-1	77	31	66	13	55	14	14	53	5.1
HA	104	5	295	59	43	75	75	53	5.4
DEAE-2	31	1	757	151	34	187	187	34	4.1
Aff-1	15	0.006	4,590	918	7	880	880	7	5.2

S/D is shikimate dehydrogenase/dehydroquinate.

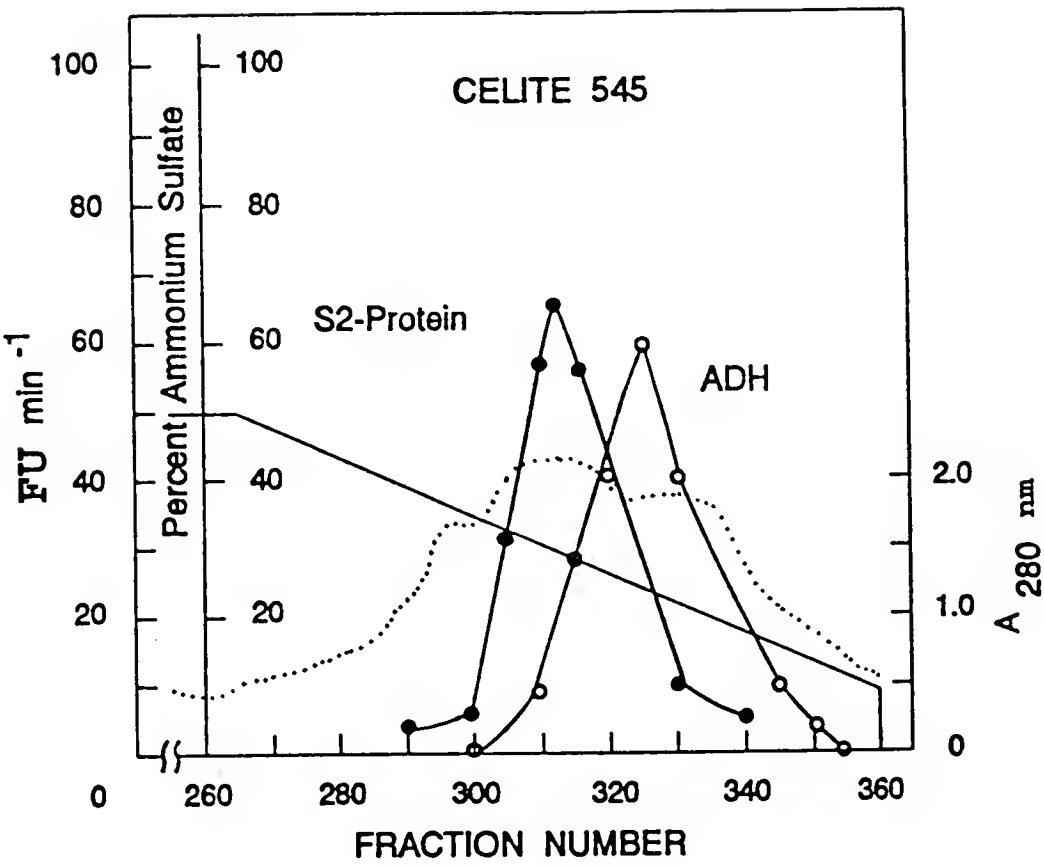


Fig. 4-4. Celite 545 column chromatography overlap of arogenate dehydrogenase and SP-II. Both SP-II (●) and ADH (O) elute in the 45 to 0% ammonium sulfate gradient (solid line) with overlapping activity peaks. Protein was monitored at $A_{280\ nm}$ (...).

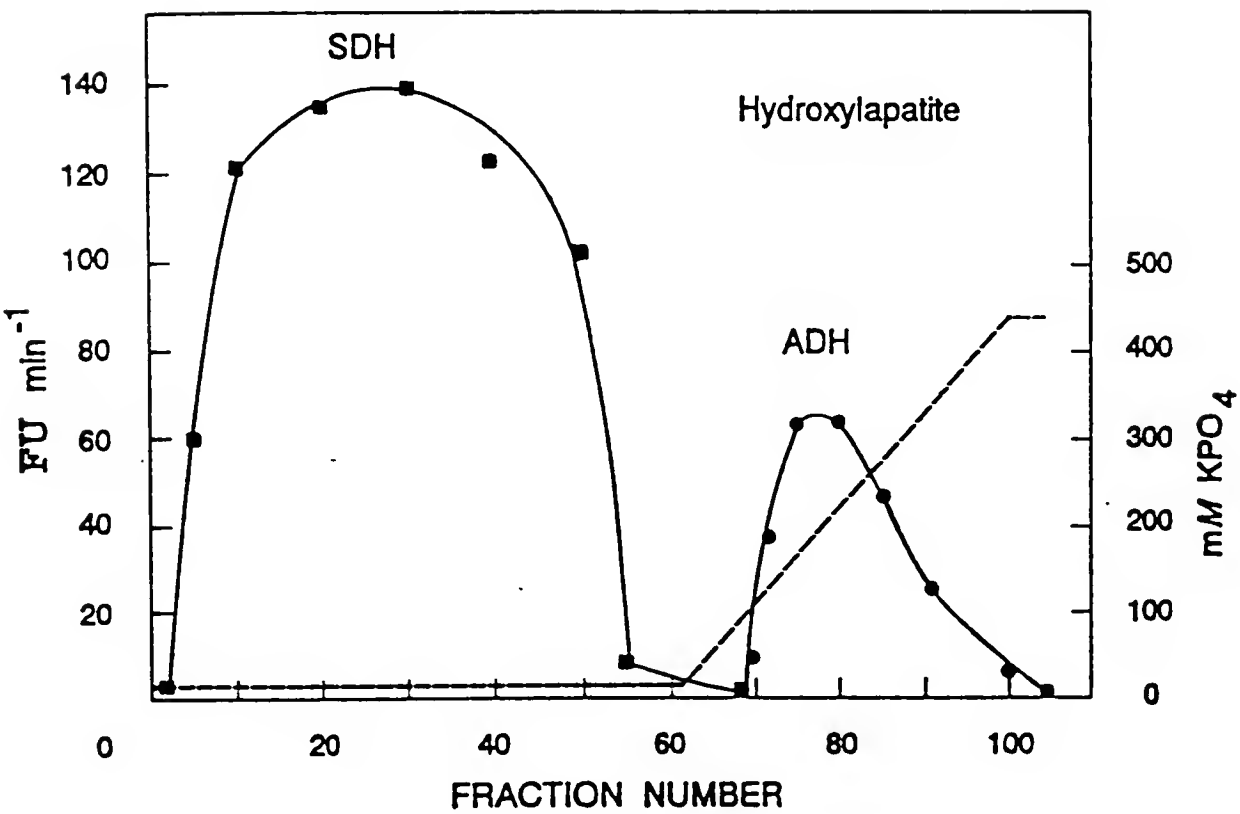


Fig. 4-5. Hydroxylapatite column chromatography separation of arogenate dehydrogenase and SP-II. The SDH activity (■) was found in the wash fractions and ADH (●) eluted at about 120 m*M* KPO_4.

in similar fractions from the $NADP^+$ affinity column. Therefore, when ADH was purified by the earlier mentioned procedure an HA column step was included to completely remove SDH activity. Purification of PAT followed the previously demonstrated procedure with a purification of about 250-fold.

Discussion

The SP-I and SP-II proteins, well separated from each other on the Celite 545 column, were each purified further to about 1100- and 900-fold, respectively, by a series of chromatographic steps. This complete separation was always repeatable under similar conditions of the Celite 545 column step (at least 12 times). SDH and DQT activities coeluted at an activity ratio of about 5 throughout the entire purification regimen for both bifunctional proteins. These proteins may be isoenzymes from different compartments (chloroplast and cytosol) or they may be isoenzymes residing in different locations within the chloroplast. This second possibility was reported for two DAHP synthase isoenzymes (DS-1 and DS-2) presumably located in chloroplasts of *Arabidopsis* (2). Further studies which may elucidate differential properties of the two proteins and localization studies should provide information with respect to possible isoenzyme species and their location within the plant cell.

The purified SP-I and SP-II proteins were studied further to characterize both the DQT and the SDH activities in (Chapter V). The purified SP-I was used to produce specific antibody against the protein (see Chapter VI) and for N-terminal sequencing (see Chapter VII). Purification of PAT and ADH was completed and then used to produce specific antibodies to each protein for use as probes in cDNA cloning.

CHAPTER V

PROPERTIES OF THE S-PROTEINS

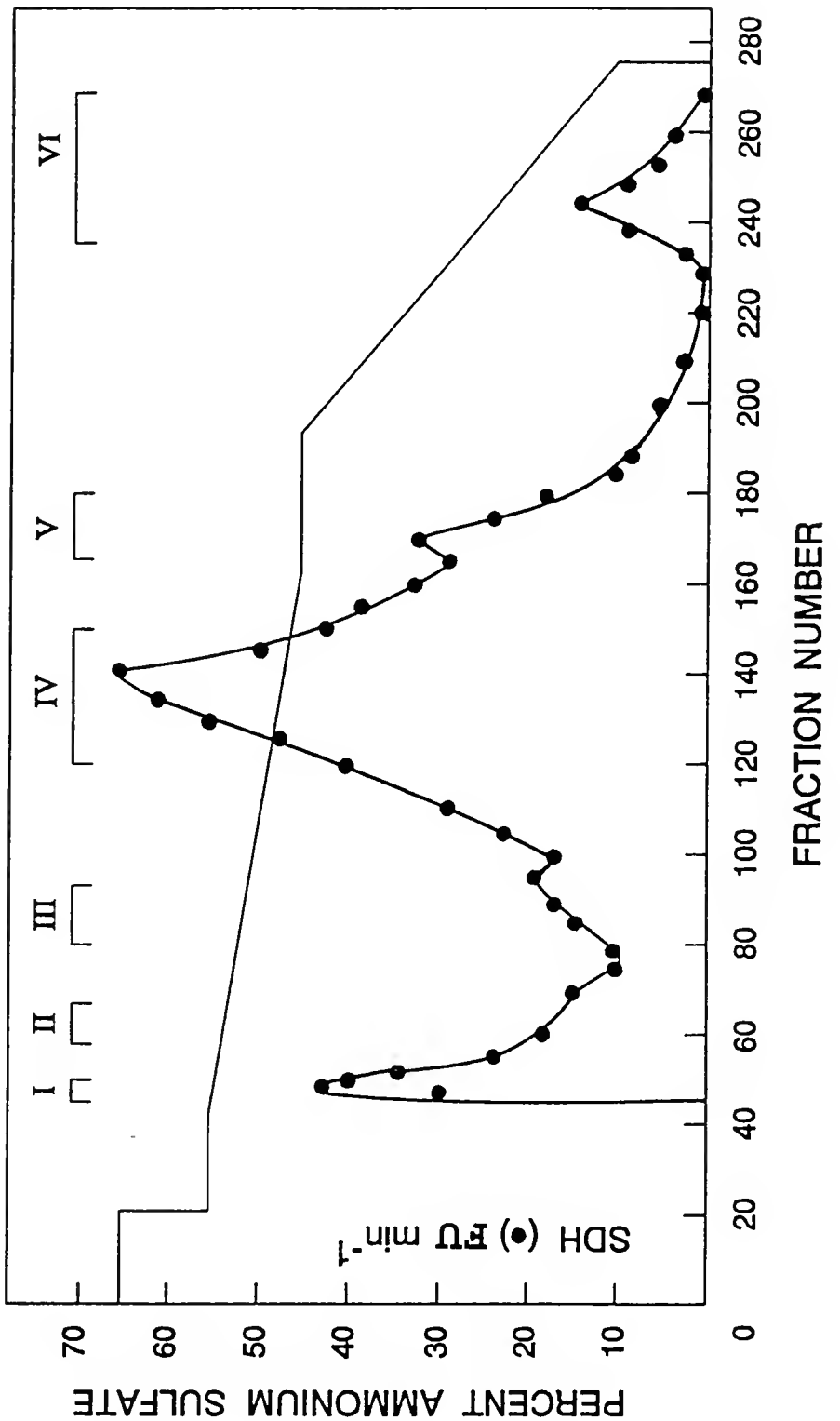
The main focus in my studies of the properties of the two bifunctional proteins was to detect possible differential properties which might suggest that these proteins are isoenzymes. Molecular mass, pH and temperature optima, thermal inactivation, and Km values for both functional domains of the bifunctional proteins, SP-I and SP-II, have been determined. Effects on activity of each protein by various compounds, including PCMB, were also studied.

RESULTS

Activity peaks on a Celite column

A Celite 545 column resolved about 6 peaks of SDH activity when a more shallow gradient of ammonium sulfate was applied than that used during the separation of the S-proteins, Chapter IV (Fig. 5-1). Dehydroquinase activity was coincident with SDH activities. Fractions of the six peaks containing the enzyme activities were pooled separately, avoiding overlapping fractions as much as possible, and were labeled as shown in Fig. 5-1. The six

Fig. 5-1. Elution profile of SDH activities from a Celite 545 column run with a shallow gradient. Five possible peaks of SDH activity eluted on a Celite 545 column with an ammonium sulfate gradient from 55% to 45% of saturation for about 600ml (labeled I to V) . A sixth peak (VI) of SDH activity eluted in a second gradient from 45% to 0% ammonium sulfate.


PERCENT AMMONIUM SULFATE
70
60
50
40
30
20
10
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
FRACTION NUMBER
SDH (•) FU min-1
I
II
III
IV
V
VI

pooled samples containing DQT/SDH activities were concentrated and examined for possible differential properties, substrate requirements for saturation with SHK and $NADP^+$, alternative substrate specificities (i.e., NAD^+ and QA), temperature optimum, thermal inactivation, SDS PAGE and activity-stained native PAGE. No significant differences were found between the overlapping peak samples of I to V (data is not shown). All six protein samples were specific for $NADP^+$ and SHK when assayed for SDH activity. Pool sample IV (or perhaps I through V) corresponds to SP-I, and Peak VI corresponded to SP-II. SP-I and SP-II exhibited similar properties except for M_r, Km and activity stained gels.

Molecular Mass

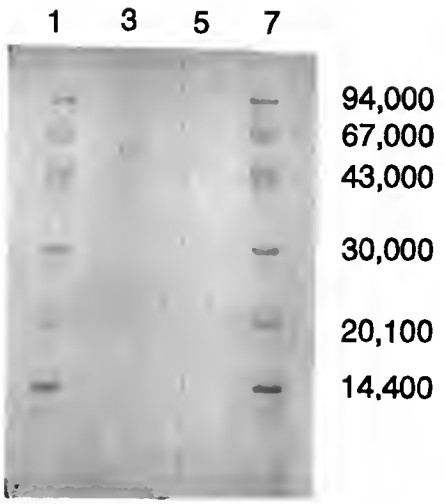
A difference was seen in the molecular mass of SP-I and SP-II as shown in Fig. 5-2. SP-I migrated slightly ahead of SP-II on SDS gels (A) and on a silver stained SDS gels (B). Both gels showed bands (or set of bands) at about 59 kD and 40 kD for purified SP-I, and bands at about 61 kD for purified SP-II. A band at 42 kD can be detected when higher concentration of SP-II was used in these experiments. The silver stained gel offered higher resolution and indicated that the affinity purified SP-I also had a set of bands at about 29 kD. Native M_r was determined on a Sephadex G-200 column,(Fig. 5-3, panels A and B), to be about 59 kD and 62 kD for SP-I and SP-II, respectively. In crude extract

Fig. 5-2. Molecular weight determinations by SDS PAGE and by silver stain of the two S-proteins. A) The SDS gel shows 2 bands for SP-I (584 ng) at molecular weights of about 60,000 and 40,000 in lane 3. Lane 5 has a single detectable band at a molecular weight of about 62,000 for the SP-II (155 ng). Lanes 1 and 7 are molecular weight markers with MW as indicated. B) The silver staining technique was also used to determine molecular weights of the S-proteins. Lanes 1, 9, and 16 show molecular weight markers. Lanes 2 through 7 represent SP-I and lanes 11 through 14 represent SP-II at various stages of purification as follows:

Lane	Protein	purification step	Molecular weight(s)		
2	S1 25ul	Affinity pure	60,000	40,000	
3	S1 10ul	Affinity pure	60,000	40,000	
4	S1 25ul	DEAE-cellulose	60,000	40,000	29,000
5	S1 10ul	DEAE-cellulose	60,000	40,000	29,000
6	S1 10ul	Affinity pure (AB)	60,000	40,000	29,000
7	S1 25ul	Affinity pure (AB)	60,000	40,000	29,000
11	S2 25ul	Affinity pure (1)	62,000		
12	S2 10ul	Affinity pure (1)	62,000		
13	S2 25ul	Affinity pure (2)	62,000		
14	S2 10ul	Affinity pure (2)	62,000		

(AB) is purified enzyme from several runs that were combined, passed through the affinity column a third time and concentrated. For SP-II the (1) represents the purified protein after the first pass through the affinity column and (2) represents combined column runs of SP-II passed a second time through the affinity column and concentrated.

A


1 3 5 7
94,000
67,000
43,000
30,000
20,100
14,400

B

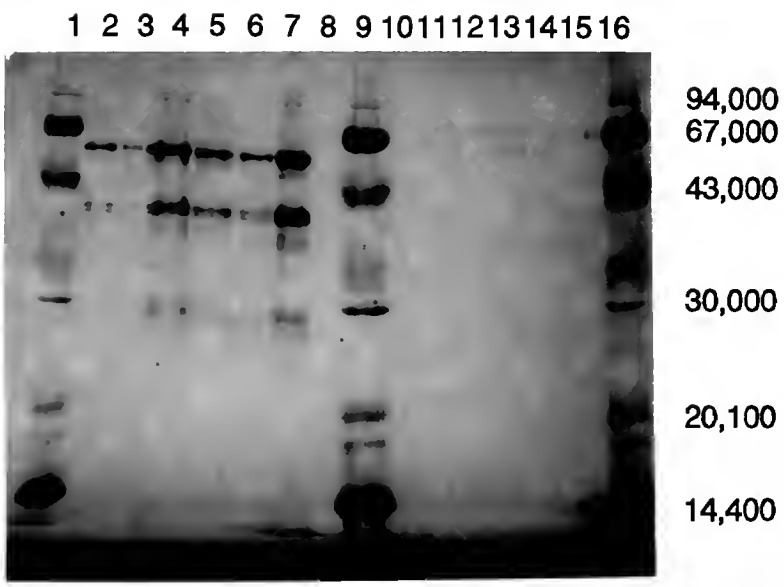
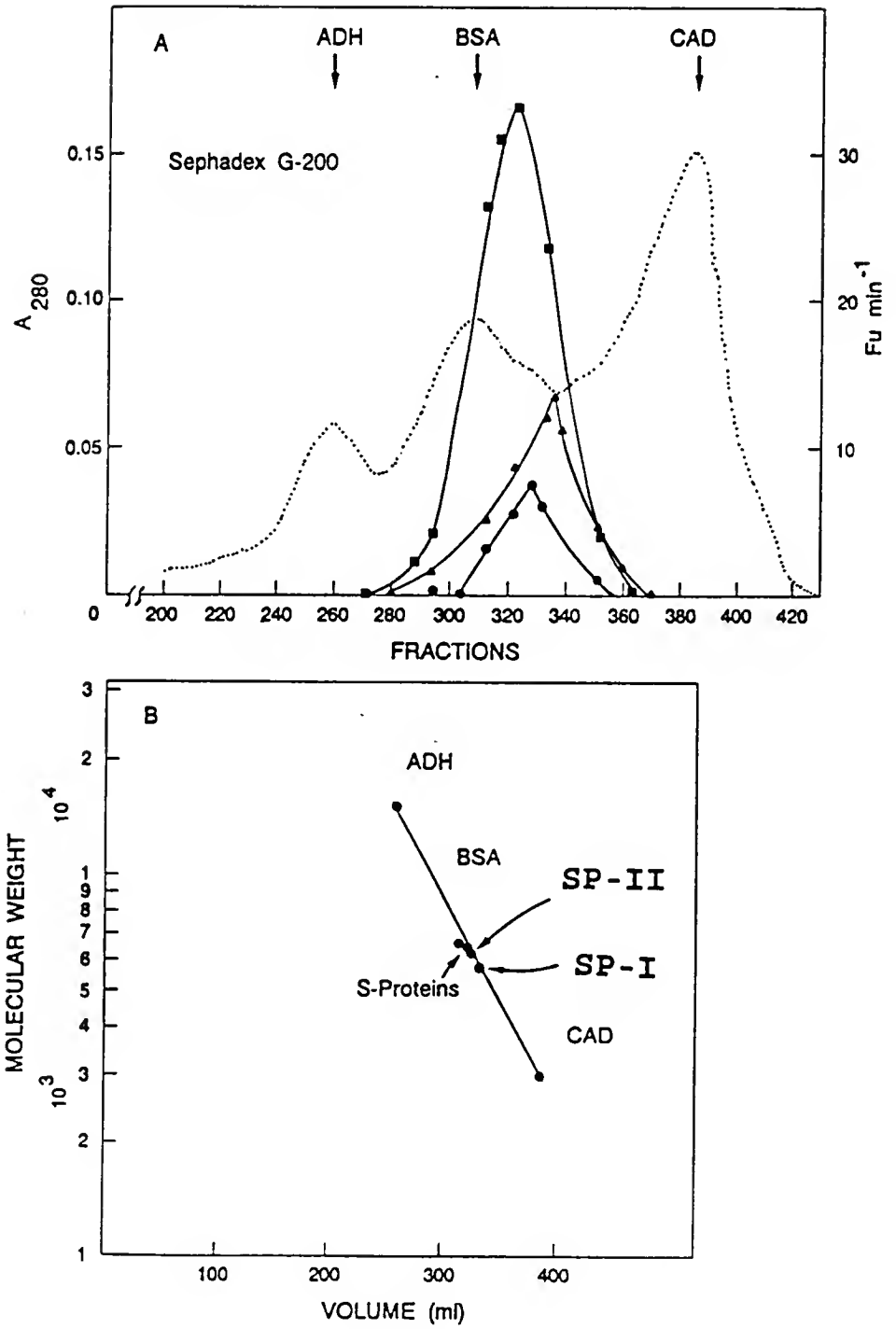

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
94,000
67,000
43,000
30,000
20,100
14,400

Fig. 5-3. Gel filtration chromatography of S-proteins. A) Concentrated crude extract (■), pool IV (SP-I) (●) and pool VI (SP-II) (▲) (from the shallow gradient Celite 545 column) were passed through a Sephadex G-200 column. The dotted profile represents the three marker enzymes (alcohol dehydrogenase, BSA and carbonic anhydrase). B) A molecular weight curve based on the column profile from A suggests the molecular weights for the SP-I to be about 59,000 and about 62,000 for SP-II. Crude extract S-protein is suggested to be about 63,000.


A
ADH
BSA
CAD
Sephadex G-200
0.15
0.10
0.05
0
A280
30
20
10
Fu min-1
200 220 240 260 280 300 320 340 360 380 400 420
FRACTIONS
B
ADH
BSA
SP-II
SP-I
S-Proteins
CAD
MOLECULAR WEIGHT
10^4
10^3
3
2
1
9
8
7
6
5
4
100
200
300
400
VOLUME (ml)

(containing all fractions of the S-protein), the bifunctional protein was determined to have only one native molecular weight of about 63 kD.

Activity stained gels

In order to optimize conditions for a native PAGE activity stain gel, various combinations of substrate and dyes were studied with concentrated crude extract, data not shown. All activity stained gels were assayed at optimal conditions as described in Methods (Chapter II). Activity stained bands required both substrate, SHK and $NADP^+$ for visibility. Crude extract revealed 3 bands on native gels after application of the activity stain (Fig. 5-4). In Fig. 5-5, purified SP-I and SP-II had 2 activity stained bands each. SP-II migrated at a faster rate than did SP-I.

pH and temperature optima of S-proteins

Both functional domains of the two bifunctional proteins were studied with respect to pH optima (Fig. 5-6). SDH activity was followed in a pH range of 6.5 to 10 in 200 m*M* Bis Trispropane buffer and showed optimal activity at pH 9.0 for both proteins. DQT, assayed by the coupled assay method had very low activities when the same buffer was used over the same pH range for both SP-I and SP-II. A pH range from 6.1 to 9.5 in potassium phosphate, Epps or glycine buffers, each at 100 m*M* resulted in higher activities and showed optimal activity at pH 7.25 for both proteins. When the three buffers above were used for the SDH assay there

Fig. 5-4. Optimal PAGE activity staining for SDH in crude extract. An activity stain of a native polyacrylimide gel was done at optimal conditions when concentrated crude extract of 360 ug was applied to the gel. Three bands are shown after incubation in substrates at concentrations for optimal activity.

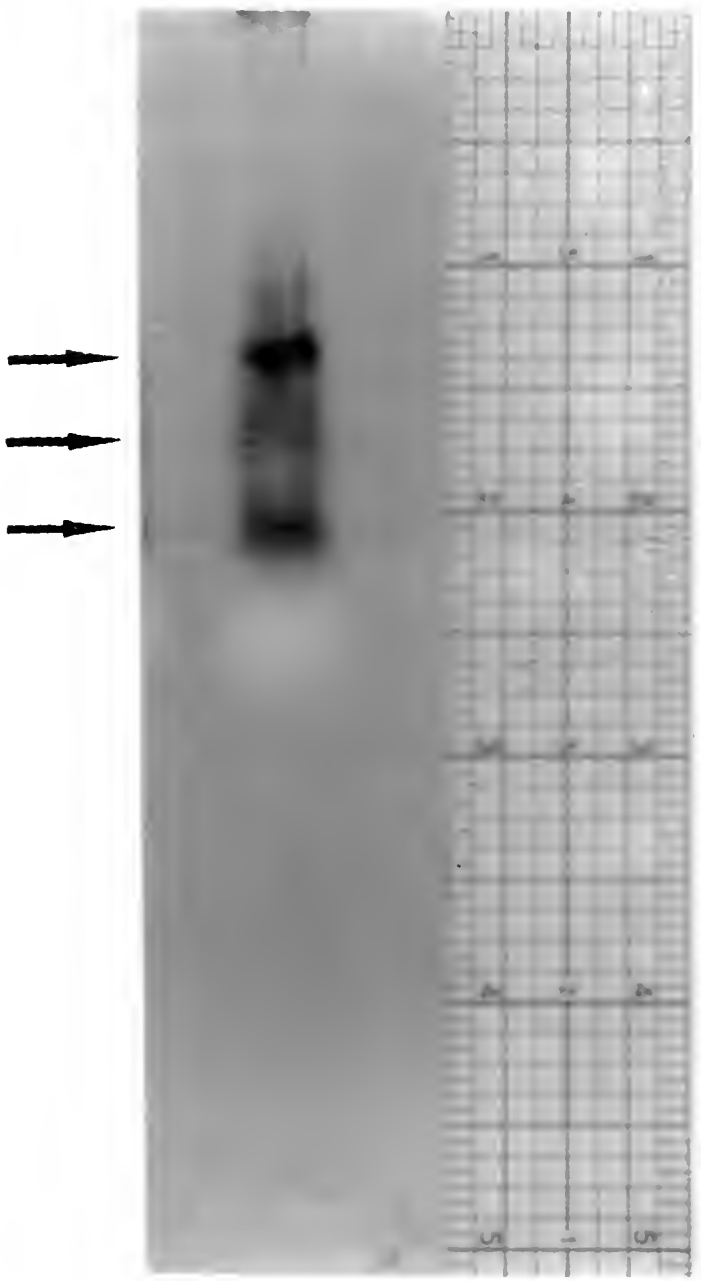


Fig. 5-5. PAGE activity staining of purified SP-I and SP-II. SP-I was added to lanes 2 and 3, and SP-II was added to lanes 1 and 4. Activity staining was done under optimal conditions. A) After incubation for about 15 min, SP-I showed two bands of activity in both lanes. SP-II, at lower concentrations, showed two bands in lane 4, each migrated faster than the SP-I bands. B) The gel was incubated further for about 15 min. The SP-II in lane 1 was then visible. C) After incubation for 1 h, 2 bands were more pronounced for all four lanes.

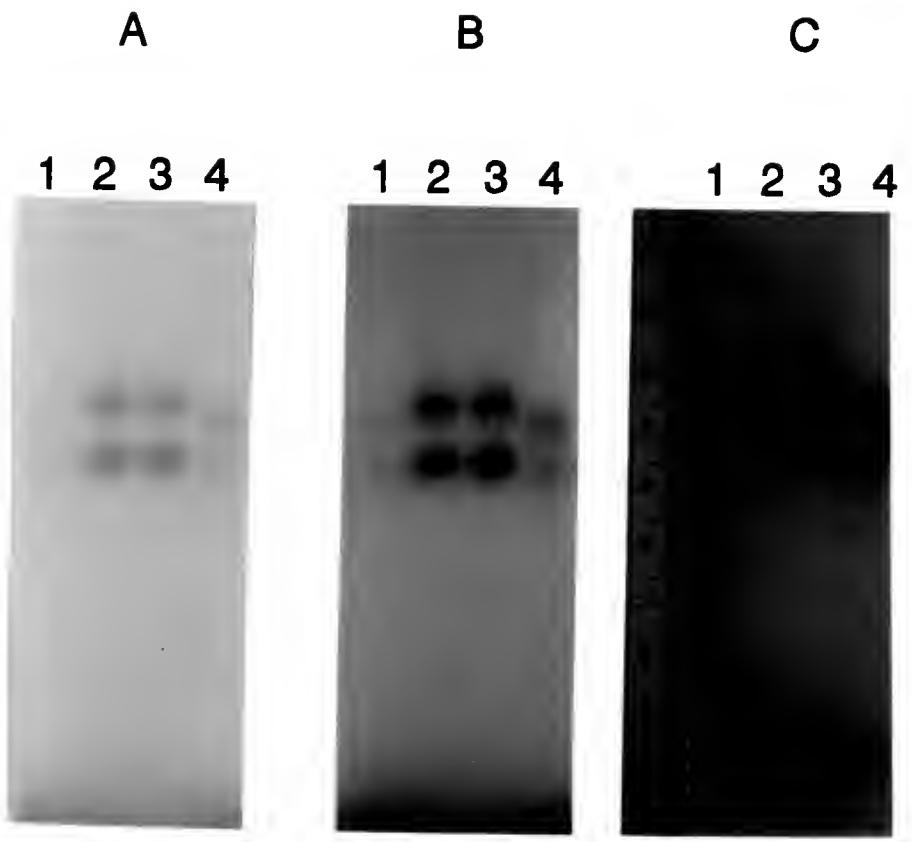
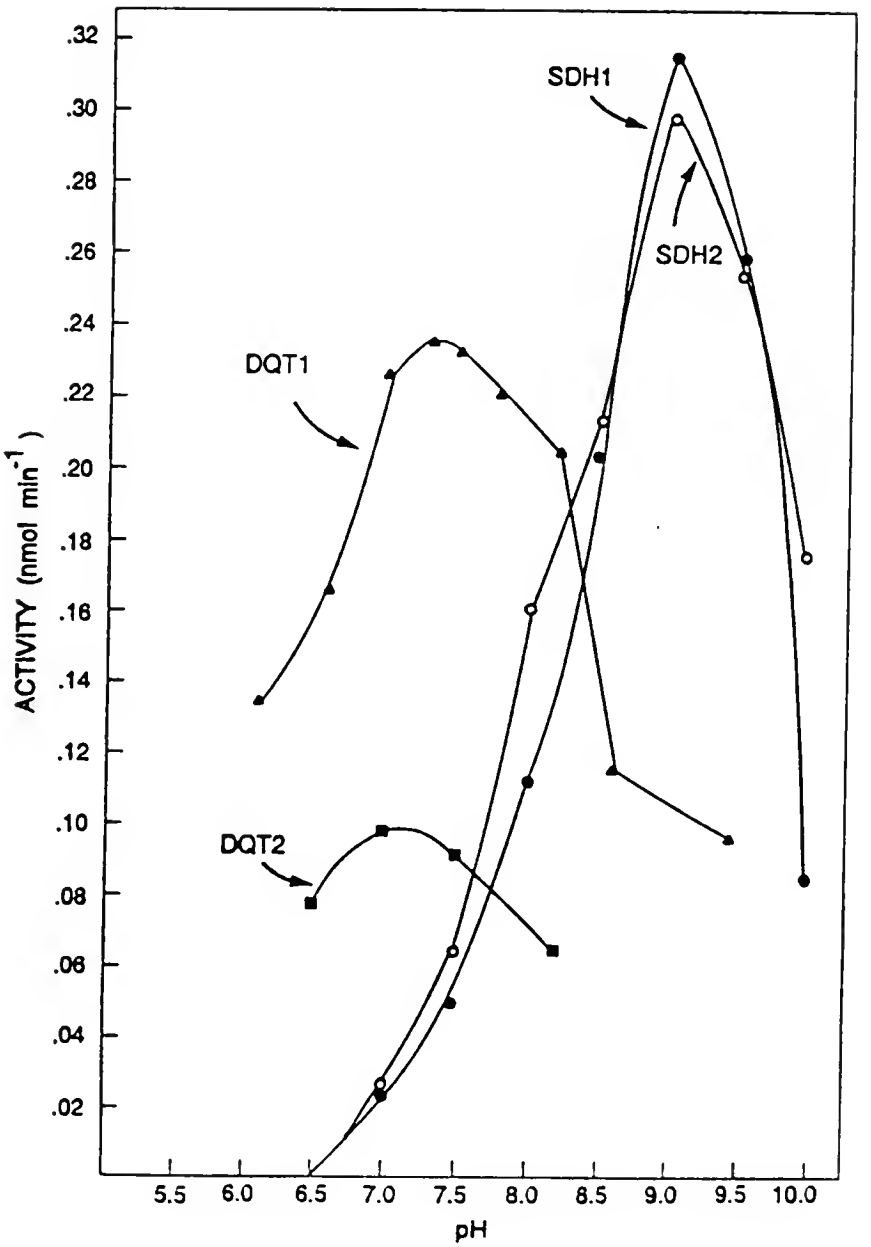

A
B
C
1 2 3 4
1 2 3 4
1 2 3 4

Fig. 5-6. S-proteins pH optima for activity. SP-I and SP-II pH optima were determined with purified enzymes (using 4ng and 8ng protein, respectively) in a range from pH 6 to pH 10. Shikimate dehydrogenase activity was determined in the presence of 200 m*M* Bis Trispropane buffer at the indicated pH values. Dehydroquinate dehydratase activity was determined with 100 m*M* potassium phosphate, Epps, or glycine buffer at appropriate pH values for each.


.32
.30
.28
.26
.24
.22
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
ACTIVITY (nmol min^{-1})
SDH1
SDH2
DQT1
DQT2
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

was no difference in activity levels from that seen with Bis Trispropane buffer.

A temperature curve was followed for each activity of the two bifunctional proteins (Fig. 5-7) using optimal assay conditions for activity. Optimal activity occurred at about 40°C for the SDH functional domain and at about 32°C for the DQT functional domain of either protein. Extract of each bifunctional protein was heated to 30°C, 40°C or 50°C for 5 to 25 min, then assayed at room temperature (24°C) as shown in Fig. 5-8. Activity decreased rapidly at all three temperatures, by 10 min of incubation at 50°C no activity was detected for either protein. Even at 30°C the enzymes were subject to inactivation. Substrate was shown to protect the functional SDH domain of both enzymes from thermal inactivation (Fig. 5-9). With the addition of $NADP^+$ prior to incubation at 50°C for 5 min, SP-I retained 67% of its SDH activity and SP-II retained about 61% of the SDH activity compared to 8% and 15% SDH activity retained, respectively, when $NADP^+$ was not added. SHK was considerably less protective against thermal inactivation with only 18% and 21% retention of SDH activity for SP-I and SP-II, respectively. Without substrate present, SP-I was thermally inactivated to 50% and SP-II to 60% of activity after 2 min at 50°C.

Fig. 5-7. Temperature optima for S-proteins. Optimal activity was determined for both SP-I and SP-II purified proteins over a range of temperatures from 24^{0}C to 66^{0}C. A) Dehydroquinate dehydratase activity was determined for the temperature range given. B) Shikimate dehydrogenase activity was determined over the given temperature range.

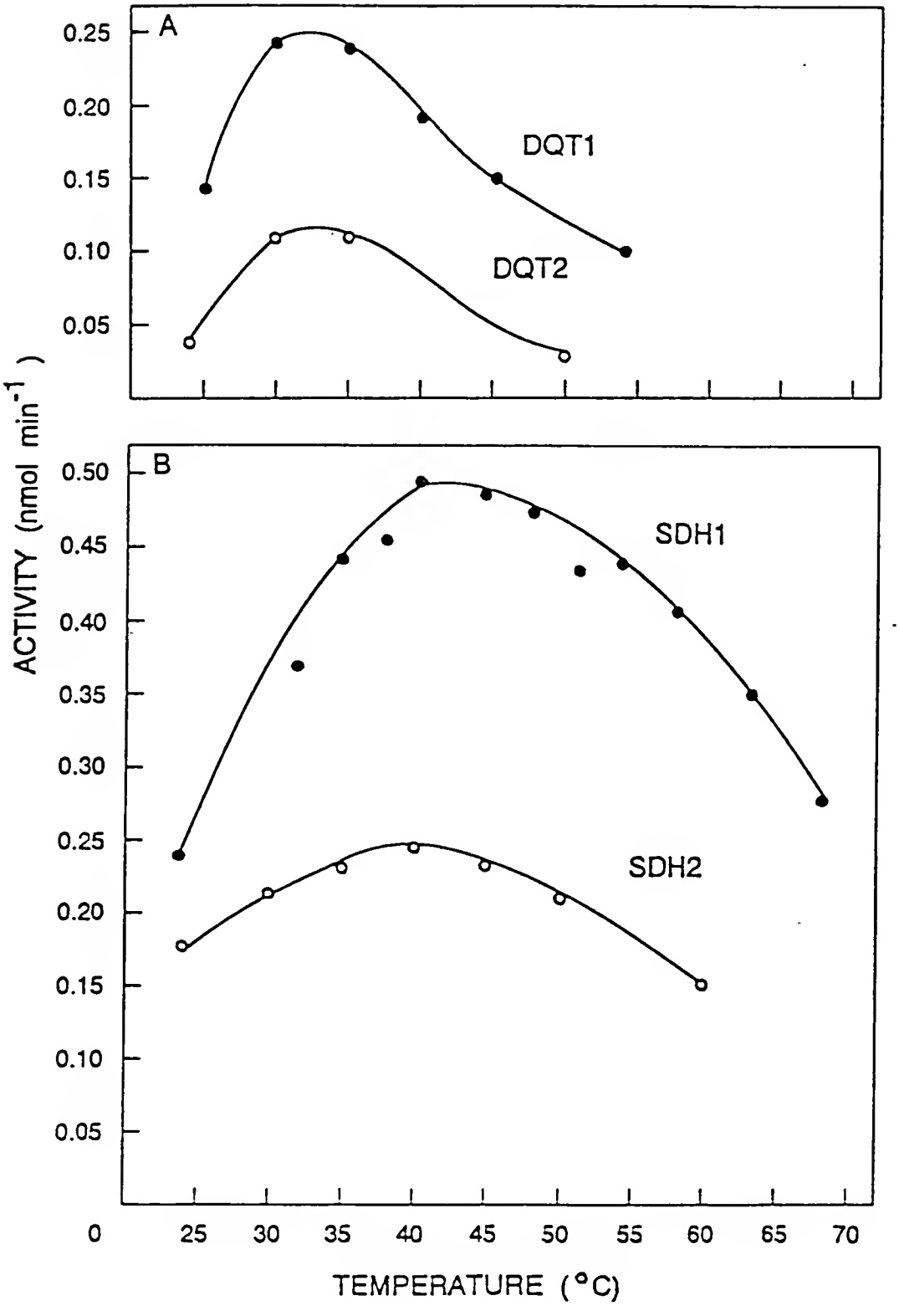

A
B
DQT1
DQT2
SDH1
SDH2
ACTIVITY (nmol min-1)
TEMPERATURE (°C)
0.25
0.20
0.15
0.10
0.05
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
25
30
35
40
45
50
55
60
65
70

Fig. 5-8. Thermal inactivation of S-proteins. Purified extracts containing the S-proteins were heated at 30°C (●),40°C (▲), or 50°C (■) from 5 to 25 min, then assayed at 25°C at saturating substrates. A) SP-I (4 ng protein) was assayed for SDH activities at various temperatures. B) SP-II (6 ng protein) was assayed for SDH activity at various temperatures.

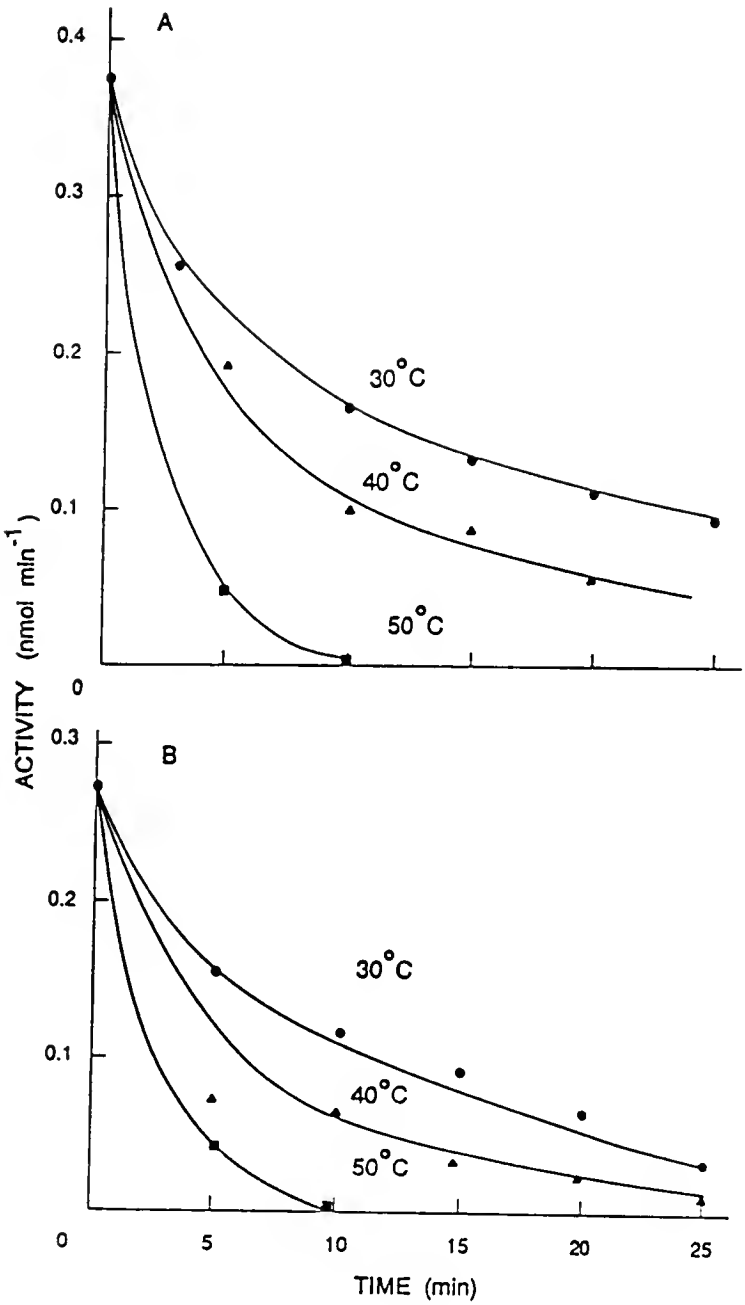
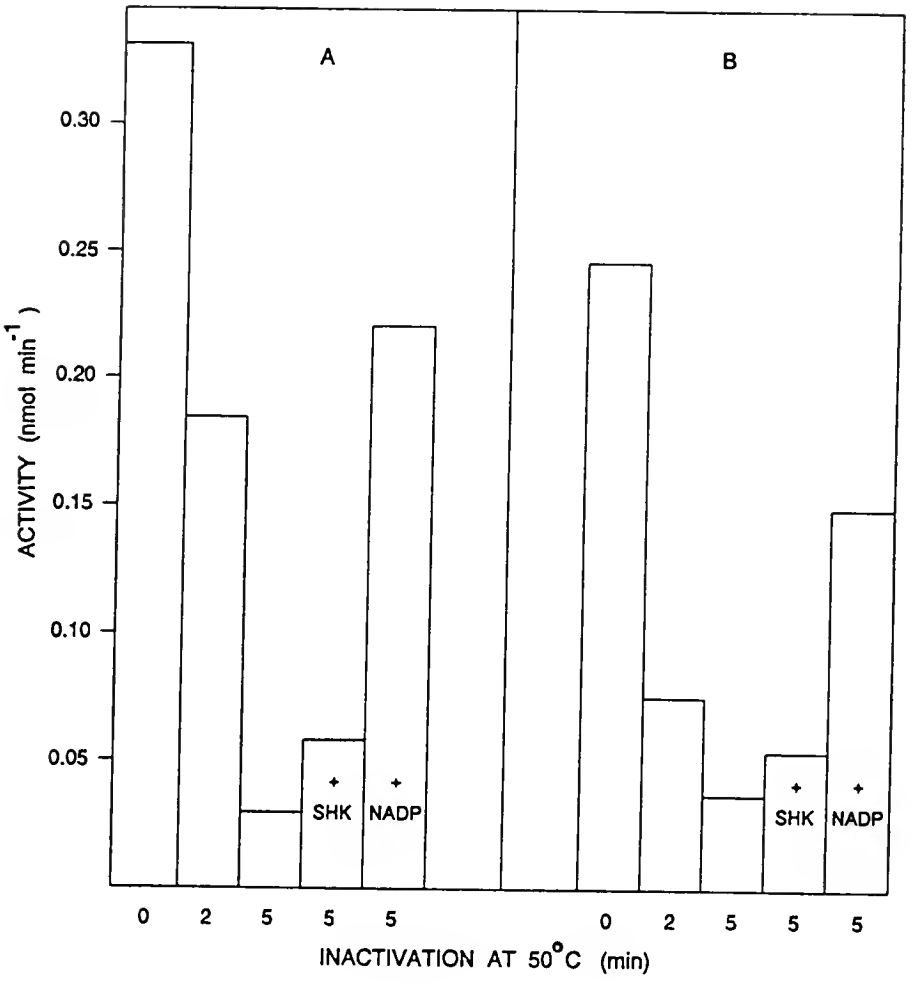

A
B
0.4
0.3
0.2
0.1
0
30°C
40°C
50°C
ACTIVITY (nmol min-1)
5
10
15
20
25
TIME (min)

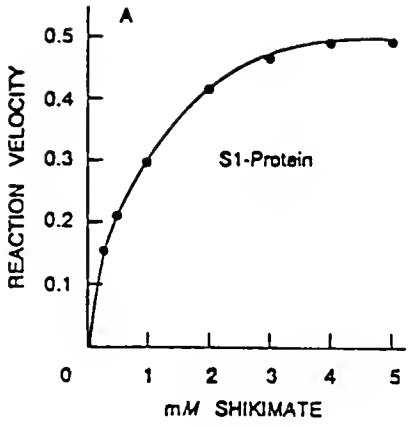
Fig. 5-9. Substrate protection against thermal inactivation of S-proteins. Purified enzyme was incubated at 50°C for 2 or 5 min without substrate added or 5 min with $NADP^+$ or SHK added, assayed at 25°C and compared to unheated extract activity. Conditions for assay were the same as described in Fig. 5-8. SP-I results are shown in panel A and SP-II results are shown in panel B.

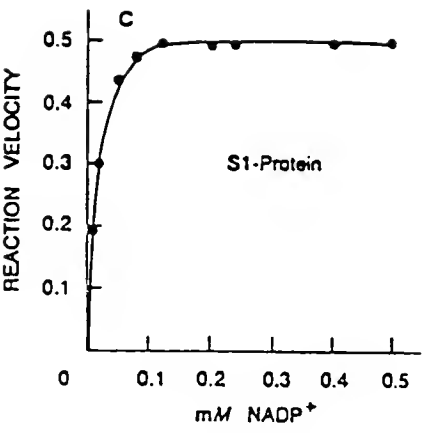

A
B
0.30
0.25
0.20
0.15
0.10
0.05
ACTIVITY (nmol min-1)
+ SHK
+ NADP
+ SHK
+ NADP
0 2 5 5 5
0 2 5 5 5
INACTIVATION AT 50°C (min)

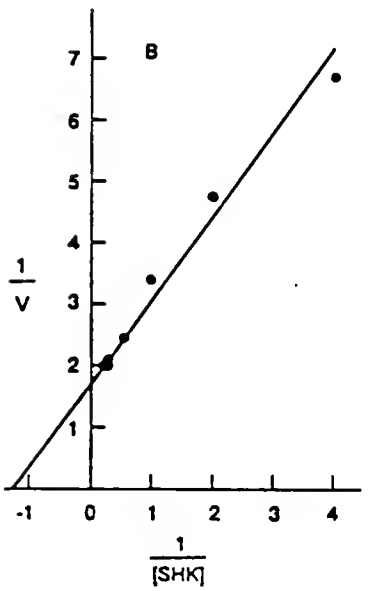
Kinetic constants

Affinity purified SP-I and SP-II were used to characterize the properties of the two proteins. Both proteins were specific for their substrates and neither enzyme would utilize NAD^+ as cofactor or quinate as substrate. Saturation of the SDH activity domain of the bifunctional enzyme with substrates, $NADP^+$ (at 0.14 m*M*) and SHK (at 5.0 m*M*), is shown in Fig. 5-10 (A and C) for SP-I and in Fig. 5-11 (A and C) for SP-II (saturating at 0.2 m*M* $NADP^+$ and 4.0 m*M* SHK. From the double-reciprocal plots in Figs. 5-10 and 5-11 (panels B and D), the Km values for the SDH domain of both SP-1 and SP-II were found to be 0.02 m*M* for $NADP^+$, However, different values for shikimate of 0.8 m*M* for SP-I and 0.36 m*M* for SP-II were obtained. Saturation curves and double-reciprocal plots were determined for both proteins (data not shown) when assayed in the forward direction with DHS and NADPH. SP-I had a Km of 0.36 m*M* with DHS and SP-II had a difference of 0.26 m*M* for DHS. The Km value for NADPH was the same for SP-I and SP-II at 0.01 m*M*. SP-I and SP-II had different Km values for DQT activity with substrate DHQ (0.07 and 0.04 m*M* DHQ, respectively, data not shown). Table 5-1 relates the Km and Vmax values and the ratio of Vmax to Km for each functional domain activity of the two proteins.

Fig. 5-10. Saturation curves and double reciprocal plots for SP-I. A) Saturation of shikimate dehydrogenase when substrate, $NADP^+$, was held constant at 0.5 m*M* and substrate, shikimate, was varied over a range of 0.25 m*M* to 5 m*M*. B) A double reciprocal plot of the data obtained in A when SHK was the variable. C) Saturation of SDH when SHK was held constant at 5 m*M* and $NADP^+$ was varied over a range of 0.01 m*M* to 0.5 m*M*. D) A double reciprocal plot of the data obtained from B when $NADP^+$ was the variable.


A
REACTION VELOCITY
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
S1-Protein
mM SHIKIMATE


C
REACTION VELOCITY
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
S1-Protein
mM NADP+


B
1/V
7
6
5
4
3
2
1
-1
0
1
2
3
4
1/[SHK]

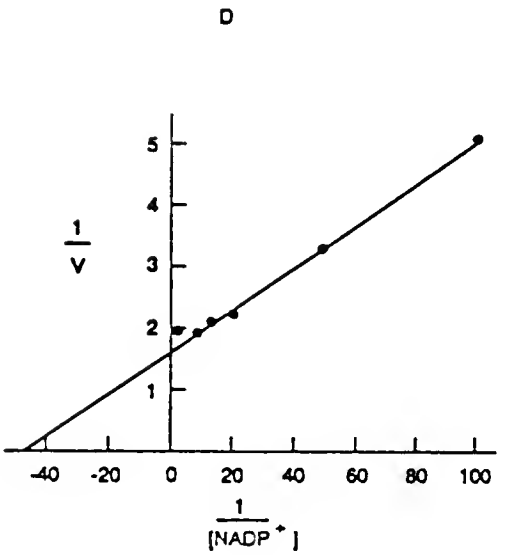
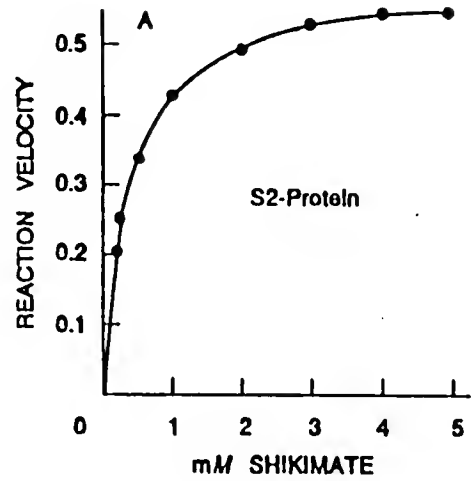
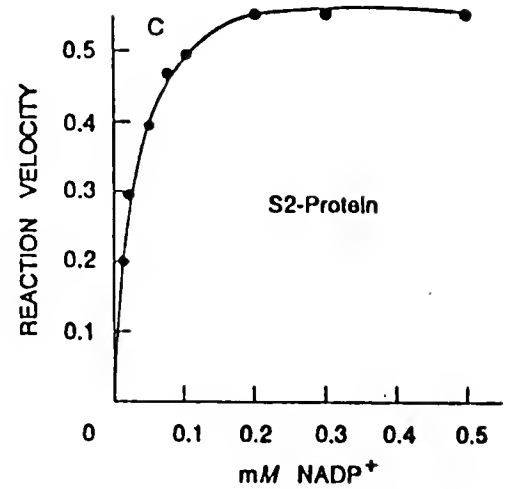
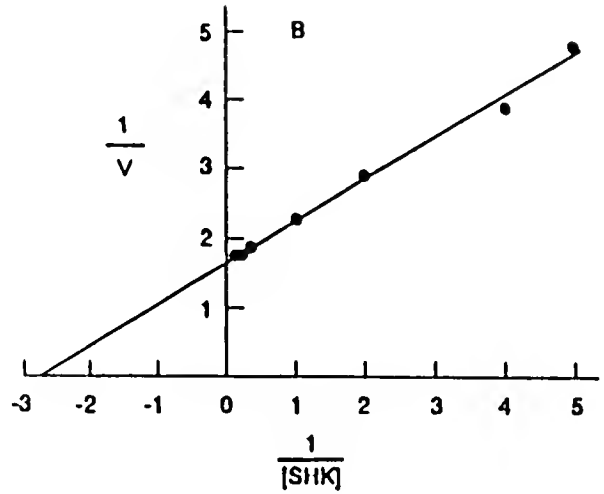

D
1/V
5
4
3
2
1
-40
-20
0
20
40
60
80
100
1/[NADP+]

Fig. 5-11. Saturation curves and double reciprocal plots for SP-II. A) Saturation of shikimate dehydrogen-ase when substrate, $NADP^+$, was held constant at 0.5 m*M* and substrate, shikimate, was varied over a range of 0.25 m*M* to 5 m*M*. B) A double reciprocal plot of the data obtained from A when SHK was the variable. C) Saturation of SDH when SHK was held constant at 5 m*M* and $NADP^+$ was varied over a range of 0.01 m*M* to 0.5 m*M*. D) A double reciprocal plot of the data obtained from B when $NADP^+$ was the variable.


A
REACTION VELOCITY
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
S2-Protein
mM SHIKIMATE


C
REACTION VELOCITY
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
S2-Protein
mM NADP+


B
1/V
5
4
3
2
1
-3
-2
-1
0
1
2
3
4
5
1/[SHK]

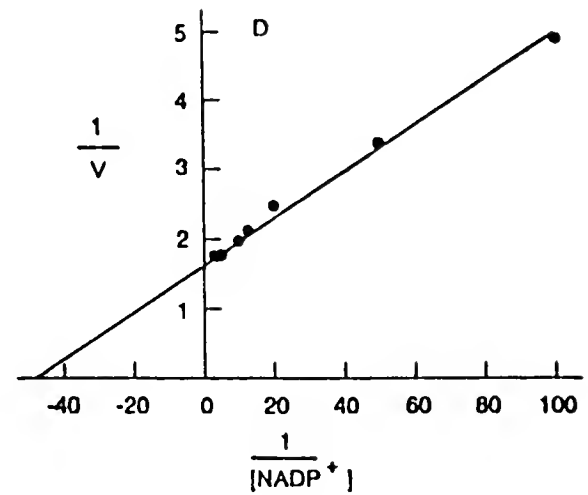

D
1/V
5
4
3
2
1
-40
-20
0
20
40
60
80
100
1/[NADP+]

Table 5-1. Kinetic parameters for SP-I and SP-II.

Enzyme		Substrate	Km (m*M*)	Vmax (nmol/min)	(Vmax/Km)
SP-I	SDH	SHK	0.80	0.60	0.75
		NADP	0.02	0.59	29.50
		DHS	0.36	0.25	0.69
		NADPH	0.01	0.26	26.00
	QDT	DQH	0.07	1.96	28.00
SP-II	SDH	SHK	0.36	0.60	1.66
		NADP	0.02	0.59	29.50
		DHS	0.26	0.27	1.04
		NADPH	0.01	0.26	26.00
	QDT	DQH	0.04	1.47	36.00

Inhibitor effects on enzyme activities

Protocatechuic acid (0.5 m*M*) inhibited SDH activity of both SP-I and SP-II at about 15% at saturating substrate concentrations (5.0 m*M* SHK and 1.0 m*M* $NADP^+$) and about 57% for each at 0.8 m*M* SHK (SP-I), 0.36 m*M* SHK (SP-II) and 0.2 m*M* $NADP^+$. PCA did not inhibit the DQT activities of the two proteins. PCMB was inhibitory to SDH activities for both functional domains and activated the DQT functional domains from both bifunctional proteins as shown in Table 5-2.

Neither SP-I or SP-II were effected by additions of 0.5 m*M* PHE, TYR, TRP, quinate, and cinnamic acid when added to saturated substrate reaction mixes and assayed for DQT or SDH activities. Metals at 0.2 m*M* concentrations (calcium, magnesium, manganese, cobalt, zinc, and iron) had no effect on activities of either protein. Incubation of DTT had no effect on any of the activities. EDTA at 10 and 125 m*M* had

no effect on activity when tested at pH 7.0, 8.6, or 9.5.

Table 5-2. Effect of PCMB on Enzyme Activities

Protein	Enzyme	mM PCMB	Inhibition	Percent 0.5 mM DTT Prevention	Percent 0.5 mM DTT Reversal	Percent Activation
SP-1	SDH	0.000	0.0	NA	NA	NA
	SDH	0.125	100	100	0.0	NA
	SDH	0.250	100	100	0.0	NA
	SDH	0.500	100	95	0.0	NA
	SDH	1.000	100	81	0.0	NA
	DQT	0.050	0.0	NA	0.0	50
	DQT	0.100	0.0	NA	NA	50
SP-II	SDH	0.000	0.0	NA	NA	NA
	SDH	0.500	100	96	0.0	NA
	DQT	0.050	0.0	NA	NA	55

DISCUSSION

A shallow ammonium sulfate gradient on a Celite 545 column revealed about 6 peaks of SDH activities. All SDH activity peaks had DQT activity as well. No differential properties were found between the five overlapping peaks containing SDH and DQT activities which eluted in the gradient from 55% to 45% of $(NH_4)_2SO_4$ saturation. These could be isoforms of SP-I or artifacts of the column chromatography. The sixth peak (also referred to as SP-II) had several differential properties when compared to SP-I which contained all of or some of the five activity peaks from the Celite 545 shallow gradient column profile. A difference of molecular mass of about 3,000 was determined

between SP-I (M_r at 59,000 to 60,000) and SP-II (M_r at about 62,000) from gel filtration, SDS and silver stained PAGE. Bands were also present at about 40,000 (SP-I) and at about 42,000 (SP-II). It was not clear what the lower molecular mass bands represent. These bands could be contaminating protein, isoforms of each protein, or proteolytically damaged protein. Activity stained gels showed three bands (each of which may contain two bands for a total of six bands) of activity for SDH from crude extracts, while, purified SP-I had two bands that migrated slower than the two bands of SP-II. The difference in M_r, may suggest these proteins are isoenzymes. One possibility, is that SP-I, the smaller M_r protein (the major fraction of activity that was located on the Celite column) may be the mature chloroplast isoenzyme with transit peptide removed and that SP-I, the larger M_r protein (the minor fraction of activity from the column) might be the cytosolic preprotein and isoenzyme that functions in aromatic biosynthesis. A second differential property, that of Km values was observed for SP-I and SP-II. SP-II has greater affinity for SHK, DHS and DHQ, substrates for both functional domain of the bifunctional protein. Both proteins have about the same affinity for cofactor, $NADP^+$ or NADPH. These Km values are in the range of Km values found in the literature (listed in Table 1-1). The difference in affinity between SP-I and SP-II for substrates is also suggestive of differentially located isoenzyme.

The Vmax/Km ratio reflects catalytic efficiency of enzymes that catalyze the same reaction in cells. SP-I and SP-II have the same Vmax, but different Kms, therefore, the Vmax/Km ratio suggests that SP-II with the lower Km , is catalytically more efficient than SP-I.

PCMB, a potent inhibitor of SDH activity in plant species (48, 53) inhibited both bifunctional proteins, implying that the presence of sulfhydryl groups are critical for catalytic activity of the proteins. This inhibition can be prevented by the addition of thiol reagents, such as DTT, which did, in fact, protect the enzyme from inhibition by PCMB. Most interesting is that PCMB activated activity of the DQT functional domain. My interpretation is that this may reflect physical overlapping of catalytic sites on the protein. Protocatechuic acid, also a known inhibitor of SDH activity (53) inhibited SDH activity of both proteins, but had no effect on the DQT functional domain. PCA is the product of the quinate catabolic system, and its physiological significance as an inhibitor of SDH is unclear.

The pH and temperature optima did not indicate any differences between the two S-protein, but were different for the functional domains of the two proteins. The SDH functional domain of the bifunctional proteins had the higher pH and temperature optima. Thermal inactivation studies of the two bifunctional protein showed that the SDH

functional domain of each protein rapidly lost activity over time even at 30°C and was protected from inactivation by cofactor much better than by substrate. From the data obtained, it appears that SP-II was stabilized to a slightly greater degree than the SP-I (about 6% greater when $NADP^+$ was present at incubation and about 4% greater for SHK). Substrate protection of the DQT functional domain of the two proteins with DHQ was not performed at this time.

CHAPTER VI

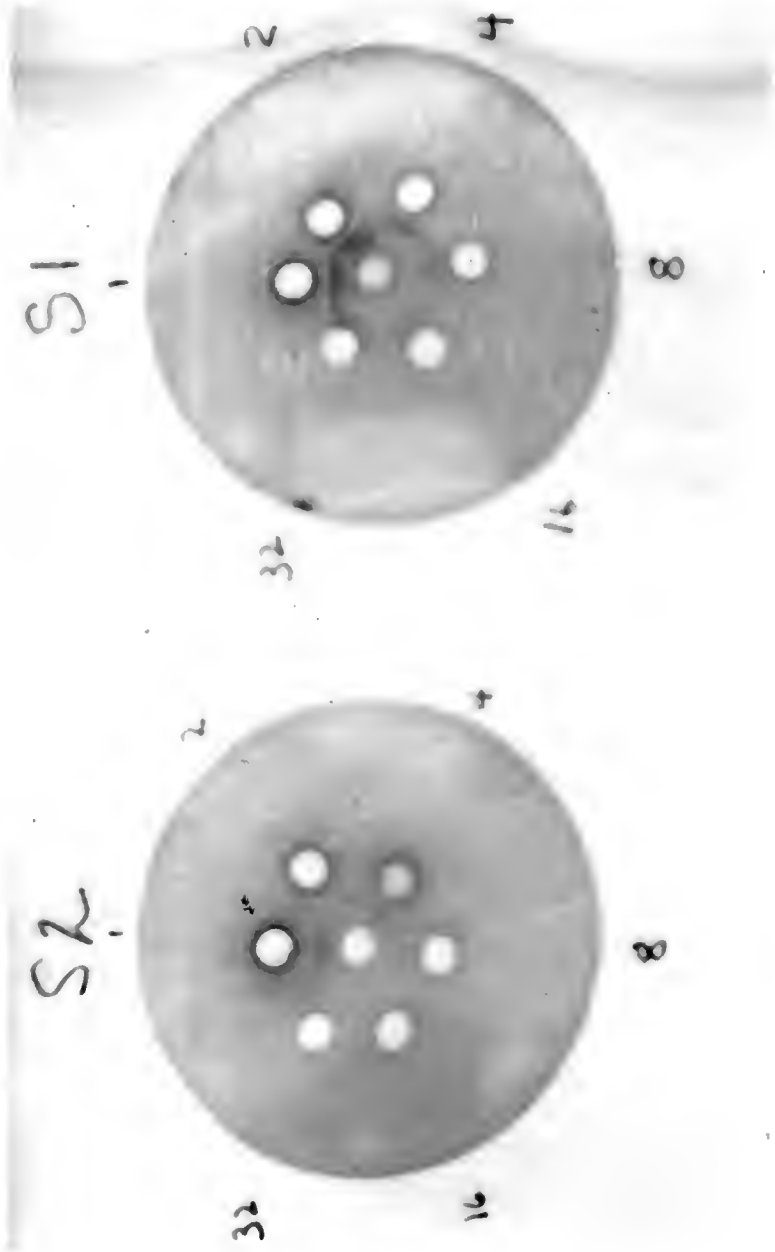
SPECIFIC ANTIBODY TO THE BIFUNCTIONAL S-PROTEIN AND TO TWO POST-PREPHENATE PROTEINS

Specific antibody was made to the purified bifunctional SP-I, to purified arogenate dehydratase and to purified prephenate aminotransferase. SP-I specific antibody was characterized with respect to both SP-I and SP-II.

Results

Antibodies made to each protein (SP-I, ADH and PAT) precipitated crude protein preparations or purified antigens on "Ouchterlony" plates. Each enzyme incubated with the appropriate antibody was precipitated and activity was lost. SP-I specific antibody precipitated both SP-I and SP-II antigen on "Ouchterlony" plates at dilutions of antibody up to 32-fold as seen in Fig. 6-1. The precipitant band was more pronounced for the SP-I plate because more highly concentrated protein was available. A saturation curve of inhibition produced by antibody was similar for both SP-I and SP-II when equal activities were established using 0.0084 μg of SP-I and 0.031 μg of SP-II (between 0.230 and 0.267 nmol min^{-}, respectively). Increasing amounts of

Fig. 6-1. Precipitation of S-proteins with SP-I specific antibody on Ouchterlony plates. SP-I specific antibody was added to the outer wells of two Ouchterlony plates, at dilutions of 1, 2, 4, 8, 16, and 32-fold. SP-I was added to the middle well of the plate marked S1 on the right and SP-II was added to the middle well of the plate marked S2 on the left.


S1
2
4
8
16
32
S2
2
4
8
16
32

antibody showed maximal inhibition of both proteins with about 100μl antibody (Fig. 6-2).

Crude extract was applied to several wells of a native gel. After electrophoretic migration, the gel was divided. One half of the gel was used for Western blotting with S1-antibody and the other half of the gel was used with SDH activity stain. Figure 6-3 shows the visible bands for each.

When SP-I specific antibody was incubated with *E. coli* crude extract and *N. silvestris* crude extract and then assayed for SDH activity, (Table 5-1) the *E. coli* SDH activity was retained, while the plant SDH was precipitated and activity was lost.

Fig. 6-2. Effect of SP-I specific antibody on SP-I and SP-II activities. SP-I antibody was added to equal activity levels of SP-1 (●) and SP-II (O), and was then incubated at 37°C for 10 min, microfuged and assayed for SDH activity.

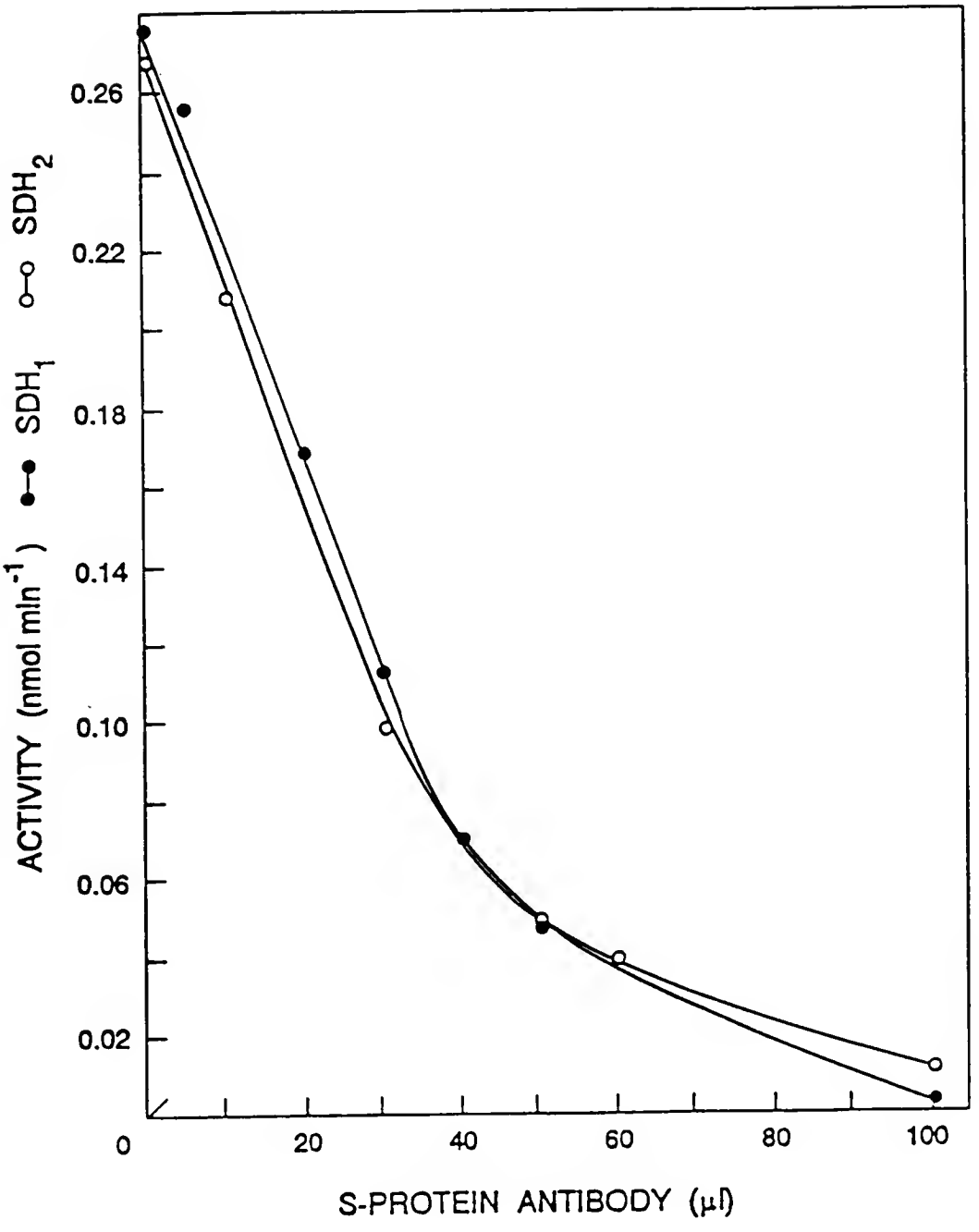

ACTIVITY (nmol mln^{-1}) ●—● SDH$_1$ ○—○ SDH$_2$
0.26
0.22
0.18
0.14
0.10
0.06
0.02
0
20
40
60
80
100
S-PROTEIN ANTIBODY (µl)

Fig. 6-3. Comparison of Western blot and activity stained gels. The number of visible bands from a Western Blot is compared to the number of visible bands from PAGE SDH specific activity stain. The native gel was loaded with crude extract from *N. silvestris* suspension cells, divided and treated (A) for a western blot using SP-I specific antibody and (B) for SDH specific activity stain.

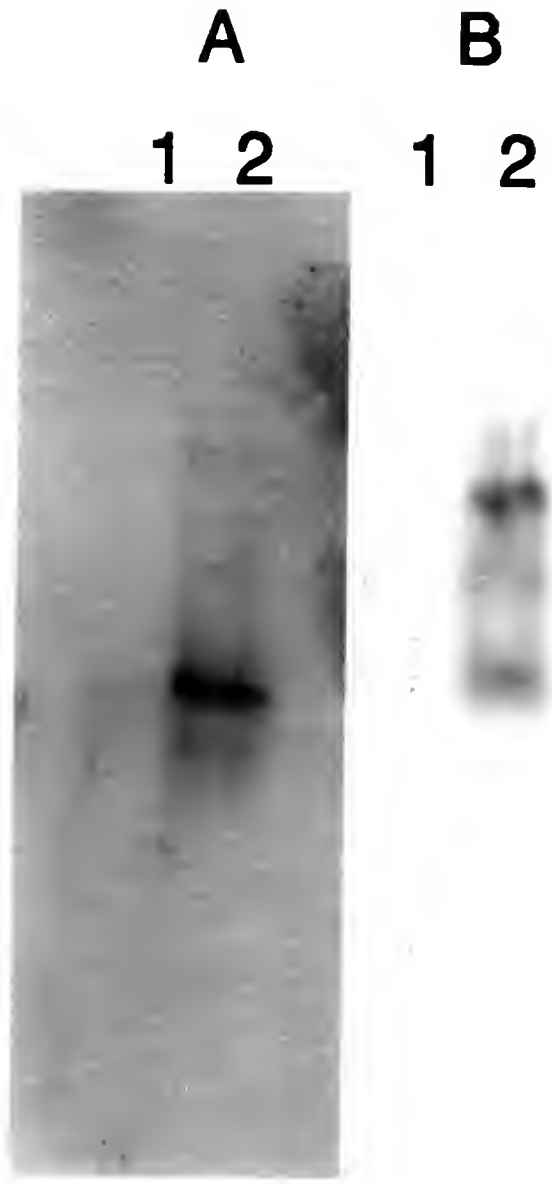

A
B
1 2
1 2

Table 6-1. S-Protein Antibody Effect on *N. silvestris* and *E. coli*[a] extracts.

		Crude Extracts (nmol min^{-1})	
Antibody (μl)	Antigen (μl)	*E. coli*	*N. silvestris*
0	25	2.73	0.364
25	25	2.72	0.031
50	25	2.73	0.016

[a]Antibody (0.16mg ml^{-1}), crude extracts (.019mg ml^{-1}) and buffer were added at a total volume of 100μl, incubated at 37°C for 10 min and microfuged before assay at 24°C.

Discussion

The two bifunctional S-proteins of *N.silvestris*, were each precipitated by antibody made to purified SP-I as seen by precipitant bands on" Ouchterlony" plates, and by loss of activity following incubation of antibody and antigen. There was no loss of activity for SDH and DQT from *E. coli* crude extracts. These results suggested that the antibody would be a suitable probe for selection of cDNA encoding for the DQT/SDH bifunctional protein of higher plants after treatment with an *E. coli* lysate to remove background contaminants. Results from a Western blot indicate the possibility of multiple SDH activities in crude extracts of *N. silvestris*. From six to seven bands may be visible on the Western blot. There are from three to six bands that are visible when an activity stain specific for SDH is applied to PAGE containing crude extract from the same protein sample. The possibility of a pentafunctional arom protein

like that found in yeast and fungi was considered, but not evident since no high molecular-weight band (would have to be in the range of about 150 kD) was found on these gels when crude extract was applied.

CHAPTER VII

CLONING cDNAS ENCODING THE BIFUNCTIONAL S-PROTEIN, ADH AND PAT

A cDNA library from *N. tabacum* was available for use in cloning cDNA encoding proteins from the aromatic amino acid biosynthetic pathway. Thus far, genes have not been cloned for the bifunctional S-protein, arogenate dehydrogenase or prephenate aminotransferase of higher plants.

RESULTS

Cloning aromatic pathway genes

S-protein cDNA. Greater than 10^6 PFU carrying inserts from a *N. tabacum* library were plated with *E. coli* XL1-Blue. Probing the library with S-protein antibody, five plaques that gave blue reactions on IPTG saturated nitrocellulose filters were designated SP3, SP5, SP6, SP10, SP33 and studied further. The size of the cDNA coding for the bifunctional protein was expected to be about 2 kB, based on molecular-weight estimations (Chapter V) and based on known sequences of yeast and *E. coli* (Table 1-1). Gel electrophoresis of purified pBluescript containing the inserted cDNAs had different mobilities on the gel (plasmid DNA was a mixture of circular and supercoiled DNA). The

expected size of the plasmid was 5 kB (2 kB insert plus about 3 kB for pBluescript). Although the size cannot be quantitated from this gel, it suggested that the DNA of the various samples were of different sizes and that the three DNA samples (SP3, SP5 and SP6) were identical in size (Fig. 7-1).

PAT and ADH cDNA. The tobacco cDNA library was prepared similarly as above to clone cDNA encoding PAT or ADH by using the specific antibody probe for each. About twelve possible clones for each were removed from plates and plaque purified. Several of the plasmids containing cDNAs appear to be of an acceptable size (Fig. 7-1, although not quantitatively determined). The expected size of cDNA encoding PAT is about 3 kB, based on a previous molecular-weight estimation of 88,000 D (7). A preliminary M_r estimation of about 130,000 for ADH by gel chromatography in *N. silvestris* (unpublished data, Bonner and Jensen, 1986) suggests that cDNA encoding ADH might be about 2.1 kB if the plant protein is a dimer (dimers from 57,600 up to 158,000 D have been described in bacteria (46, 56). Figure 7-1 shows the plasmid DNA containing possible clones.

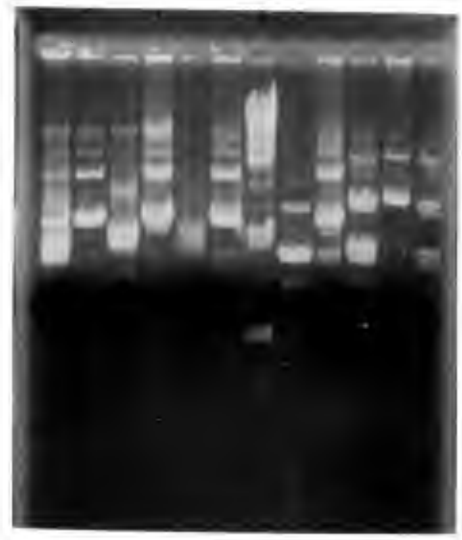
Functional complementation

Both *aroD* (DQT^-) and *aroE* (SDH^-) mutants transformed with SP3, SP5 or SP6 yielded transformants which grew on M9 medium without addition of aromatic amino acids. This result was a strong indication that all three cDNA clones

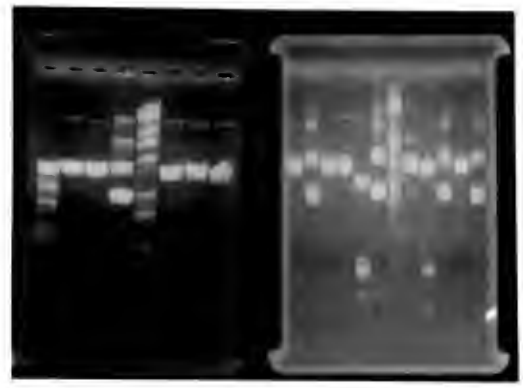
Fig. 7-1. Agarose gels of cDNA encoding aromatic pathway proteins. A) The cDNA inserts in pBluescript are shown. B and C) Restriction enzymes (all found in the multiple cloning site of pUC 18, pGEM or pBluescript plasmids) were individually used to determine the number of RE sites (or fragments) of the cloned cDNA insert in pBluescript encoding the S-protein:

A		(left) B		(right)	
Lanes	cDNA Clones	Restriction Enzyme	Sites	Restriction Enzyme	Sites
1	PAT5	HindIII	3	SalI	1
2	SP10	SacI	0	BamHI	0
3	SP33	KpnI	0	NdeI	1
4	SP3	SP3	-	SpeI	0
5	PAT7	STD	-	BanII	3
6	SP5	XbaI	0	SP3	-
7	STD	XhoI	0	STD	-
8	pBluescript	NotI	0	EcoRV	3
9	SP6			EcoRI	1
10	ADH1			SmaI	1
11	ADH4			PstI	0
12	ADH8			BbuI(SphI)	1

A



B



must encode an intact bifunctional S-protein. On the other hand, SP10 and SP33 transformants would not grow on M9 medium unless aromatic amino acids were supplied. Table 7-1 shows results obtained by direct enzyme assays of transformants carrying an *aroD•aroE* insert, in comparison to appropriate controls. These data show that expression levels of both DQT and SDH are about 15-fold higher than the wild type *E. coli* levels which have the corresponding activities.

Table 7-1. SDH/DQT activities in transformed *aroD* and *aroE* mutant strains of *E. coli*.

Enzyme Preparation			Specific Activities DQT	SDH
aroD	AT1360		0	1.3
aroD	AT1360	(+SP3)	15.0	18.7
aroE	Sk494		0.7	0
aroE	SK494	(+SP3)	11.4	17.9

Initial sequence analysis of cDNA clones

Analysis of the sequenced 5′ and 3′ ends of the 5 possible SP-clones, by the GCG Blast computer program, revealed that one clone, SP10 was $NADP^+$-specific malate dehydrogenase and that SP33 was $NADP^+$-specific cinnamyl-alcohol dehydrogenase (about 100% identity). The three remaining clones, SP3, SP5, and SP6 were identical. The 5′

end (444 bases sequenced from the T3 promoter) was translated to yield a sequence having about 23 to 25% identity with the DQT functional domain of the pentafunctional amino acid sequence from *S. cerevisiae* or *A. nidulans*, respectively, and about 22% identity with the monofunctional AroD sequence from *E. coli*, Fig. 7-2. The sequence determination at the 3' end (sequenced from the T7 promoter) was not informative at this stage because the deduced amino acid sequence showed very little conservation between the known genes of *E. coli*, *S. cerevisiae* or *A. nidulans* at the C-terminal region.

Partial sequencing of 5' and 3' ends of several of the possible ADH and PAT cDNA clones showed no significant identity to known genes.

Subcloning cDNA encoding the S-protein

Since the partial sequence at the 5'end of the cloned insert, the positive functional complementation results and the direct assay of activities were all mutually reinforcing that the cDNA encoding the S-protein had been cloned, the SP3 clone was used further for subcloning analysis in order to obtain a complete nucleotide sequence.

Based on the multiple cloning sites of the vectors to be used for subcloning, pUC18, pGEM5Zf(+) and pBluescript+, restriction enzymes were chosen and tested in order to locate and map the restriction sites present in the SP3 cDNA clone. Analysis of agarose gels (Fig. 7-1B, right and

```
                          * * *                          * ** * **
Ec AroD          41 dfDilEwRvDhya   53          75 kPlLFTfRsakEGGe   89
En AroM        1075 GsDaVElRvDlLK 1087        1113 LPiiFTiRtqsqGGr 1127
Sc Aro1        1099 GceaVEVRvDhLa 1111        1131 iPiiFTvRtmkqGGn 1145
clone SP3,
  SP5, SP6       57 GaDlVEVRlDsLK   69          85 LPtLFTyRptwEGGg   99
```

Fig. 7-2. Preliminary alignment of translated sequences of clone SP3 with established DQT sequences. The deduced N-terminal amino acid sequence of three cDNA clones, SP3, SP5, and SP6 displayed two highly conserved regions with known amino acid sequences from *E. coli* (AroD), *E. nidulans* (AroM) and *S. cerevisiae* (Aro1). All amino acids showing identity with the SP3 clone in each group are in caps. Amino acids conserved in all four proteins are noted with an asterisk.

left) indicated that HindIII, BanII and EcoRV had at least several restriction sites within the cDNA insert, and that SphI and NdeI, each had one restriction site located within the insert. The remaining restriction enzymes shown in Fig. 7-1B, only had one site within the multiple cloning region of the plasmid. From this information and from the known RE sites of the 3′ and 5′ sequenced ends of the cDNA clone (determined by GCG Map analysis) a strategy for subcloning was begun and continued as information about RE sites was obtained through subcloning (Fig. 7-3).

After appropriate RE were used to obtain fragments of suitable size for sequencing, the bands were excised from agarose gels (Fig. 7-4A) and prepared for transformation.

To confirm that the transformed cells carried plasmids containing the appropriate fragments, a cracking procedure (Promega protocol, Madison, WI), followed by an agarose gel was performed (Fig. 7-4B). This gel showed that the plasmids contained inserts of the correct size and were ready to be purified for sequencing. Agarose gels containing the purified plasmid DNA carrying the various fragment sizes are shown in Fig. 7-4C.

After sequencing the cDNA fragments on an available Li-Cor sequencer, overlapping areas were placed together to yield a complete cDNA sequence. For correction of sequencing errors, multiple sequencing of all fragments was done in both directions. The complete nucleotide sequence and the deduced

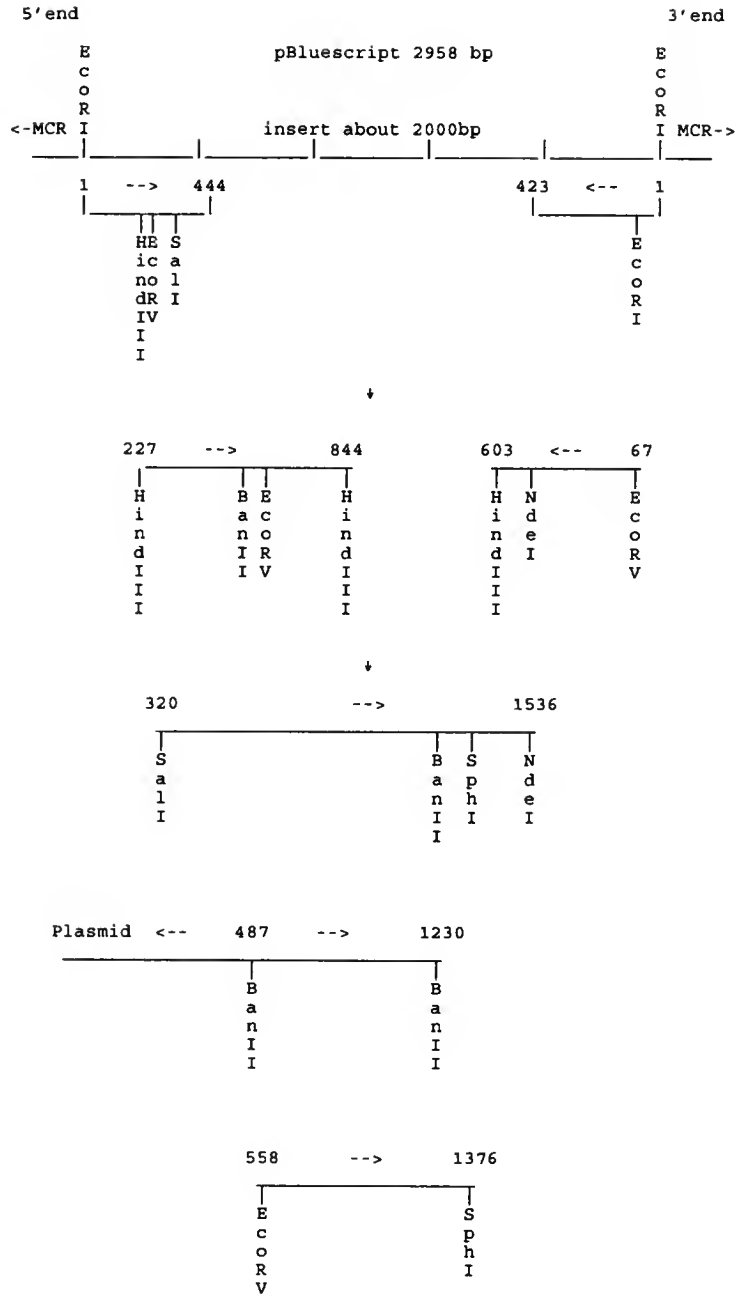
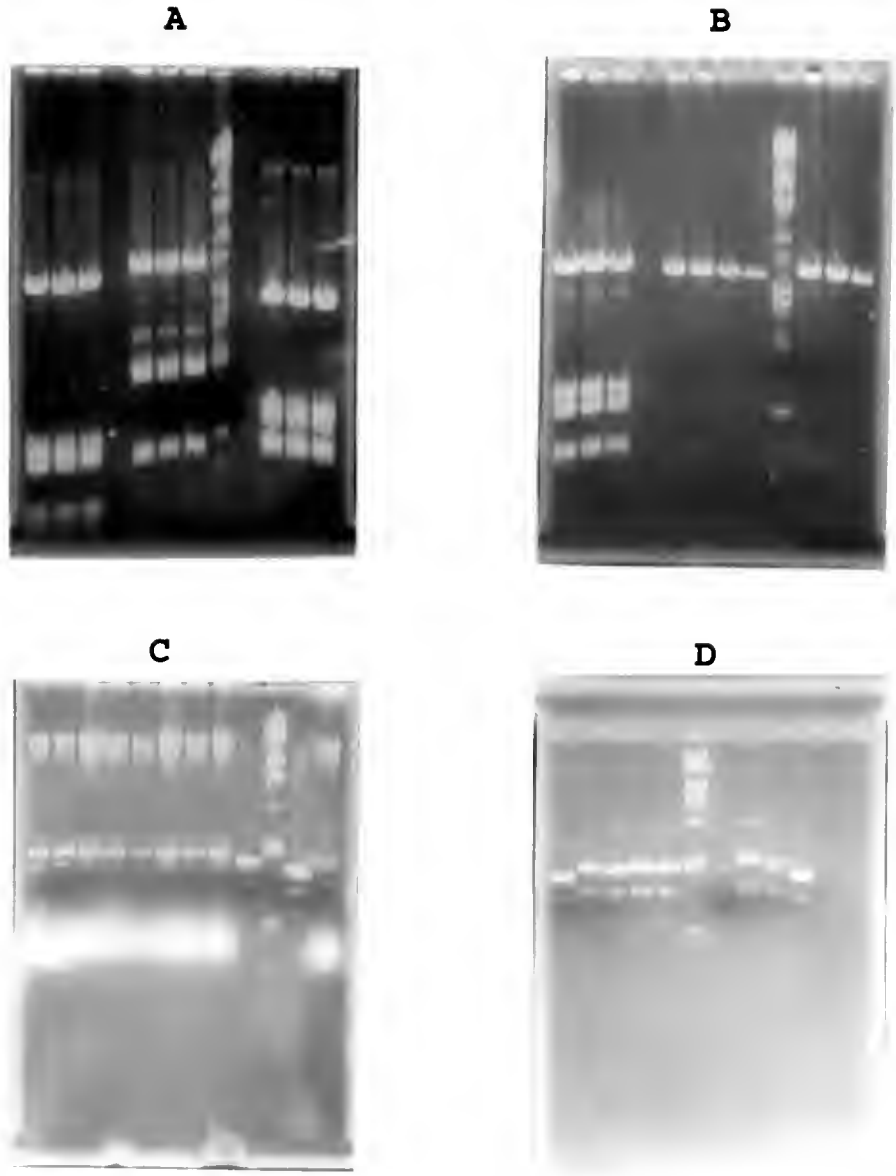


Fig. 7-3. Sequencing strategy for cDNA encoding the bifunctional protein. Overlapping fragments were obtained when the SP3 cDNA clone was cut with restriction enzymes in the sizes denoted in the Figure. The cDNA clone was first cut with *Hind* III only, and *Hind* III plus *Eco*RV. From this four of six possible fragments were obtained for sequencing, two are shown in the second step of the sequencing strategy. Next the cDNA clone was cut with *Ban* II and two of three fragment were obtained. Two combinations of RE were used, *Nde*I plus *Sal* I, and *Sph*I plus *Eco*RV. Sequencing was completed for multiple samples of these fragments in both directions.

Fig. 7-4. Agarose gels of SP3 cDNA fragments during stages of subcloning. SP3 fragments were visualized on agarose gels after incubation with restriction enzyme(s). A) The first three lanes contained cDNA fragments cut with *Hind*III (from 500 to about 800 bp), the second set of three lanes contained cDNA fragments cut with *Sph*I and *Sal*I (about 300 and 1100 bp) and the last set of three lanes contained fragments cut with *Ban*II (about 500,700 and 900 bp). B) The first three lanes contained cDNA fragments cut with *Nde*I and *Eco*RV (about 300, 500 and 900 bp). C) All lanes show that plasmids carrying the cDNA fragments were transformed into competent cells. The lowest bands represent RNA, the mid bands are the plasmid DNA bands and the top bands are genomic DNA. D) Lanes contain purified plasmid DNA containing cDNA fragments. Bands are of different sizes depending on the size of the ligated fragment.


A
B
C
D

amino acid sequence is shown in Fig. 7-5. The sequence has an untranslated 3′ end of 277 bases following a TAA stop codon and has three to four probable polyadenylation sites prior to the beginning of a nine base poly A-tail.

N-terminal amino acid sequence

The first 7 N-terminal amino acid residues of purified SP-I were found to be G E A M T R N. These amino acids corresponded to the deduced amino acid sequence starting at position number 24 (underlined in Fig. 7-5).

Analysis of cDNA coding for the S-protein

GCG sequence analysis. The GCG sequence analysis program (26) was used for analysis of the presumed mature S-protein cDNA sequence starting at amino acid residue number 24. The overall isoelectric point was 5.96 for the bifunctional protein (isoelectric points of 5.04 for the AroD functional domain and 6.99 for the AroE functional domain). A M_r of 60,388 (556 amino acids) was determined for the proposed mature protein (M_r of 28,515, 260 amino acids was determined for the AroD functional domain and M_r of 34,651, 320 amino acids for the AroE functional domain). There were no remarkable differences in amino acid composition when the AroD and AroE domains were compared. The bias of codon usage was similar to that of other higher plant genes, with A and T being favored in the third position of most alternative codons. Rare codons were TGC (one), CGC (zero), CCG (zero), and ACG (two).

Fig. 7-5. Nucleotide and deduced amino acid sequence of cDNA coding for the bifunctional S-protein. The nucleotide sequence of the cDNA encoding the S-protein, containing 2041 bases, is numbered on the right. The deduced 679 amino acid sequence is numbered on the left. The N-terminal sequenced amino acids are in bold and underlined. The stop codon is designated by the asterisk and four possible polyadenylation sites are underlined with hashed marks.

```
    CCATTTTTGTGCTCTACAAGTTGGTTGCTAATGGAGTTGGTAGTGGATTCAGGGGTGAGG   60
  1 P  F  L  C  S  T  S  N  L  L  M  E  L  V  V  D  S  G  V  R

    AAGATGGAGGGGGAGGCAATGACGAGGAACGAAACACTAATTTGTGCACCAATCATGGCA  120
 21 K  M  E  G  E  A  M  T  R  M  E  T  L  I  C  A  P  I  M  A

    GACACAGTGGATCAAATGTTGAATCTAATGCAAAAGGCTAAAATTAGTGGTGCTGATCTT  180
 41 D  T  V  D  Q  M  L  N  L  M  Q  K  A  R  I  S  G  A  D  L

    GTGGAAGTTCGATTGGATAGCTTGAAAAGCTTTAATCCTCAATCAGATATCGATACTATT  240
 61 V  E  V  R  L  D  S  L  K  S  F  N  P  Q  S  D  I  D  T  I

    ATCAAACAGTCCCCTTTGCCTACCCTTTTCACTTACAGGCCCACTTGGGAAGGGGGTCAG  300
 81 I  K  Q  S  P  L  P  T  L  F  T  Y  R  P  T  M  E  G  G  Q

    TATGCTGGTGATGAAGTGAGTCGACTGGATGCACTTCGAGTAGCAATGGAGTTGGGAGCT  360
101 Y  A  G  D  E  V  S  R  L  D  A  L  R  V  A  M  E  L  G  A

    GATTACATTGATGTTGAGCTAAAGGCTATTGACGAGTTCAATACTGCTCTACATGGAAAT  420
121 D  Y  I  D  V  E  L  K  A  I  D  E  F  N  T  A  L  H  G  N

    AAATCAGCAAAATGCAAAGTTATTGTTTCTTCTCACAACTATGATAATACACCATCATCT  480
141 K  S  A  K  C  K  V  I  V  S  S  H  N  Y  D  N  T  P  S  S

    GAGGAGCTCGGCAATCTAGTAGCAAGAATACAGGCATCTGGAGCTGACATTGTGAAGTTT  540
161 E  E  L  G  N  L  V  A  R  I  Q  A  S  G  A  D  I  V  K  F

    GCAACAACTGCACTGGATATCATGGATGTTGCACGTGTATTCCAAATTACTGTACATTCT  600
181 A  T  T  A  L  D  I  M  D  V  A  R  V  F  Q  I  T  V  H  S

    CAAGTACCAATAATAGCCATGGTCATGGGAGAGAAGGGTTTGATGTCTCGAATACTTTGT  660
201 Q  V  P  I  I  A  N  V  M  G  E  K  G  L  M  S  R  I  L  C

    CCAAAATTTGGTGGATACCTCACATTTGGTACTCTTGAAGTGGGAAAGGTTTCGGCTCCT  720
221 P  K  F  G  G  Y  L  T  F  G  T  L  E  V  G  K  V  S  A  P

    GGGCAACCAACAATTAAAGATCTTTTGAATATATACAATTTCAGACAGTTGGGACCAGAT  780
241 G  Q  P  T  I  K  D  L  L  N  I  Y  N  F  R  Q  L  G  P  D

    ACCAGAATATTTGGCATTATCGGGAAGCCTGTTAGCCATAGCAAATCACCTTTATTGTAT  840
261 T  R  I  F  G  I  I  G  K  P  V  S  H  S  K  S  F  L  L  Y

    AATGAAGCTTTCAGATCAGTTGGGTTTAATGGTGTTTATATGCCTTTGCTGGTTGATGAT  900
281 N  E  A  F  R  S  V  G  F  N  G  V  Y  M  P  L  L  V  D  D

    GTTGCAAATTTCTTTCGGACTTACTCATCTTTAGATTTTGCTGGCTCAGCTGTAACAATT  960
301 V  A  N  F  F  R  T  Y  S  S  L  D  F  A  G  S  A  V  T  I

    CCTCACAAGGAAGCCATTGTTGACTGCTGTGATGAGTTGAATCCTACCGCTAAAGTAATA 1020
321 P  H  K  E  A  I  V  D  C  C  D  E  L  N  P  T  A  K  V  I

    GGGGCTGTCAATTGTGTCGTAAGCCGACTCGATGGGAAGTTGTTTGGTTGCAATACAGAC 1080
341 G  A  V  N  C  V  V  S  R  L  D  G  K  L  F  G  C  N  T  D

    TATGTGGGTGCAATCTCCGCCATTGAAGAAGCGTTGCAAGGCTCACAGCCTAGTATGTCT 1140
361 Y  V  G  A  I  S  A  I  E  E  A  L  Q  G  S  Q  P  S  M  S

    GGGTCTCCCTTAGCTGGTAAATTATTTGTGGTCATTGGTGCTGGTGGCGCTGGCAAGGCA 1200
381 G  S  P  L  A  G  K  L  P  V  V  I  G  A  G  G  A  G  K  A

    CTTGCTTATGGTGCAAAGGAAAAGGGGGCTCGGGTGGTGATTGCTAACCGTACCTATGAA 1260
401 L  A  Y  G  A  K  E  K  G  A  R  V  V  I  A  N  R  T  Y  E

    CGAGCGAGAGAACTTGCTGATGTAGTTGGAGGTCAGGCTTTGTCTCTTGACGAGCTTAGC 1320
421 R  A  R  E  L  A  D  V  V  G  G  Q  A  L  S  L  D  E  L  S

    AATTTCCATCCAGAAAATGACATGATTCTTGCAAATACCACCTCCATTGGCATGCAACCA 1380
441 N  F  N  P  E  N  D  M  I  L  A  N  T  T  S  I  G  M  Q  P

    AAGGTTGATGATACACCAATCTTTAAGGAAGCTTTGAGGTACTACTCACTTGTATTTGAT 1440
461 K  V  D  D  T  P  I  F  K  E  A  L  R  Y  Y  S  L  V  F  D

    GCTGTTTATACGCCCAAAATCACTAGACTCTTGCGGGAAGCTCACGAGAGTGGAGTAAAA 1500
481 A  V  Y  T  P  K  I  T  R  L  L  R  E  A  H  E  S  G  V  K

    ATTGTAACAGGAGTTGAAATGTTTATCGGCCAGGCATATGAACAATATGAGAGATTTACA 1560
501 I  V  T  G  V  E  M  F  I  G  Q  A  Y  E  Q  Y  E  R  F  T

    GGGCTTGCCAGCTCCAAAGGAACTTTTCAAGAAAATTATGGCTGGATATTGAGAGCAAGG 1620
521 G  L  A  S  E  K  G  T  F  Q  E  N  Y  G  W  I  L  R  A  R

    TCTCTTTCCCTTTTCAATGCGGCCCTGCTAGTTACTTTTCCTCCTAAATCCCTACATAGT 1680
541 S  L  S  L  F  N  A  A  L  L  V  T  F  P  P  K  S  L  H  S

    TGTGTGATAGCAATGGTCTTAGATTCCTCTGCCCTACCATTTGTGCTTCGGAGGAATTAA 1740
561 C  V  I  A  N  V  L  D  S  S  A  L  F  F  V  L  R  R  N  *

    TTCGTTCCAGGTAAATCGTGATTTTCACCAAAACAAATTCTTGAGGATGTTCAGGAAGGC 1800

    AGTCAGACATACCAGTGGACAATCGCCGTCATTCTGGCTTATATTAGACTCTTGTAGCAC 1860

    TTCATTCTTTGACAACTATGGTATCTCTAATTGTGCTTTCATTAAACACAGATGTATCAG 1920

    TGTTTCTCATTGTGACCCCATACTTGGAATTCCTCTTGATTATCATTATTATTAAATCTT 1980
                                          ----- -----
    GTCACATTATTGTATGATTTGTATCAAAAAAAAA          -----          2041
     -----
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Discussion

S-protein cDNA cloning

From five possible cDNA clones isolated by antibody screening of a Lambda Zap II cDNA library from *N. tabacum*, three identical clones encoding the bifunctional S-protein, were obtained. Subcloning analysis and sequencing of the entire cDNA of clone SP3 was performed.

The other two cDNA clones, probably code for two other $NADP^+$-dependent plant proteins, malate dehydrogenase and cinnamyl-alcohol dehydrogenase. Antibody selection of these two cDNA clones could be due to the presence of these proteins as contaminants in the purified SDH/DQT bifunctional protein. Consistent with this, both MDH and CDH have very similar calculated isoelectric points (5.78 and 6.07, respectively) compared to the mature S-protein isoelectric point of 5.96. Neither CDH nor MDH activity could be detected in the purified S-protein preparation, even at high protein concentration. Alternatively, MDH and CDH might have common epitopes recognized by the S-protein specific antibody. As a final possibility, the rabbit may have developed an immune response separate from that developed to the injected protein, perhaps by ingestion of some plant similar to tobacco. Using the GAP analysis in the GCG program, the SDH functional domain of the bifunctional protein had 21% and 23% identities with CDH and

MDH, respectively. MDH had about 51% similarity with the SDH functional domain. These proteins did not show any striking conservation of residues aligned with the SDH functional domain and its homologues, except for a region thought to be important for $NADP^+$ binding.

Possible cDNAs coding for PAT or ADH

Of the number of possible cDNA clones isolated with specific antibodies to either PAT or ADH, no determinations have been made as to whether the cDNAs of interest have been cloned. The terminal regions (5′ and 3′) of the cDNAs that were sequenced did not offer enough information. Neither PAT nor ADH have been sequenced in any organism, so that comparisons of homologues are not possible at present. Although many aminotransferases have been cloned from plant, animal and microbial species, and some very highly conserved regions have been determined, they all seem to be located toward the center of the genes, with very little conservation at either terminus. There are no auxotrophic mutants available to attempt functional complimentation for these putative cDNA clones. Extract preparation and enzyme assay may help to identify the correct clones if the cDNAs are intact and functional.

Homology relationships of the DQT domain

A multiple alignment is shown in Fig. 7-6 of the AroD functional domain of the S-protein (denoted Nta-AroD•E) with its homologues. The corresponding dendrogram is shown in

Fig. 7-6. Multiple amino acid alignment of the AroD domain of AroD•E with its homologues. Nine proteins have been aligned and conserved regions are boxed. Three conserved residues that have been shown as active site residues in *E. coli* have asterisks (H (25), L (20), M (47)}. The amino acid residue numbers for each sequence are on the right of the figure:

Designation	Organism	Protein	Accession Number
Eco-AroD	*Escherichia coli*	dehydroquinase	S14750
Sty-AroD	*Salmonella typhimurium*	dehydroquinase	P24670
Bsu-AroD	*Bacillus subtilus*	dehydroquinase	L09228
Efa-AroD	*Enterococcus faecalis*	dehydroquinase	L23802
Nta-AroD•E	*Nicotiana tabacum*	bifunctional S-protein	-
Sce-Aro1	*Saccharomyces cerivisiae*	arom pentafunctional protein	P08566
Eni-AroM	*Emericella nidulans*	arom pentafunctional protein	P07547
Ncr-Qa-1S	*Neurospora crassa*	quinate repressor protein	P11637
Eni-QutR	*Emericella nidulans*	quinate repressor protein	M59935

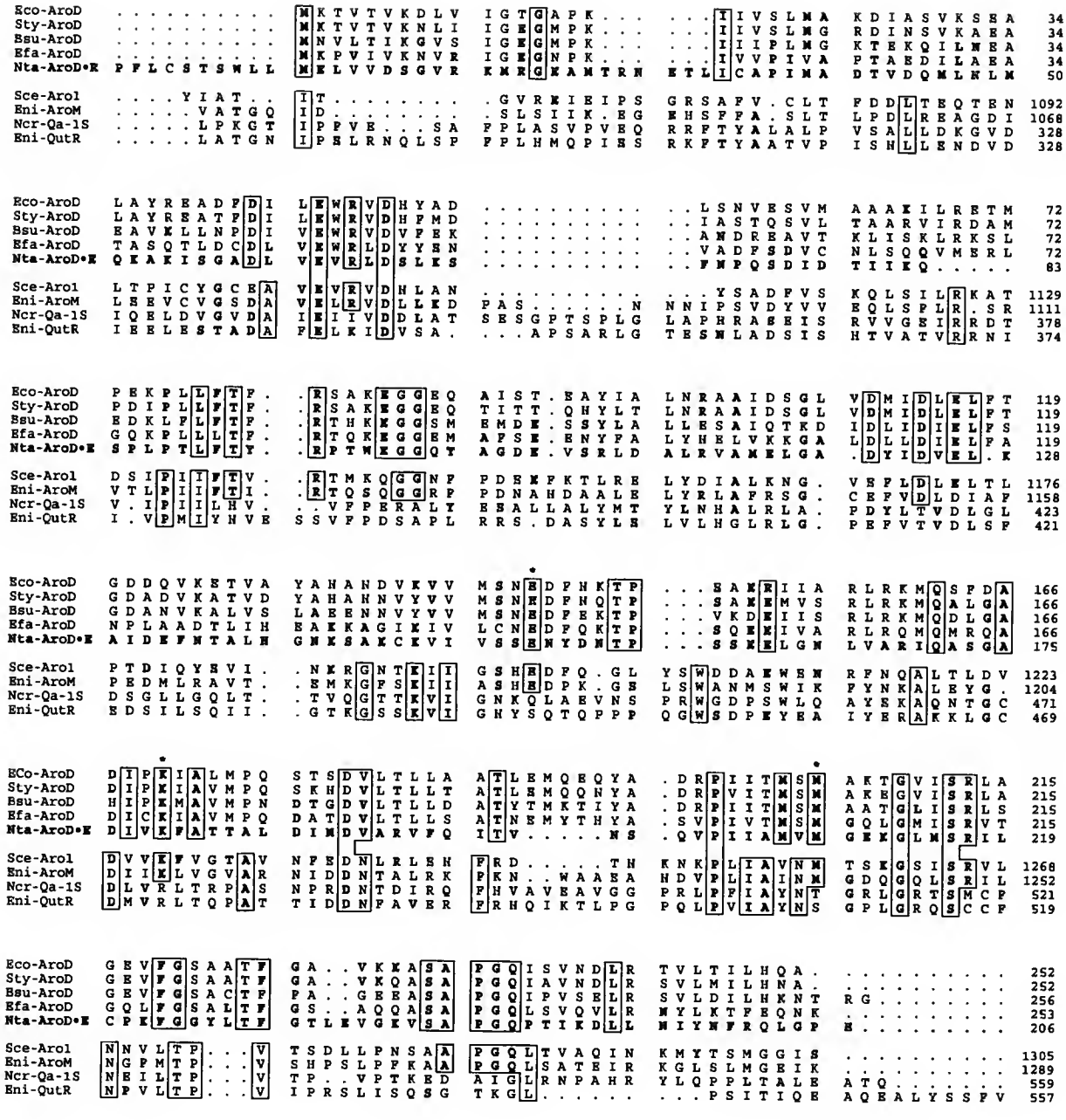


Fig. 7-7. Dendrogram of dehydroquinase homologues. The dendrogram is based upon the multiple alignment which includes the nine designated proteins explained in the legend of Fig. 7-6.

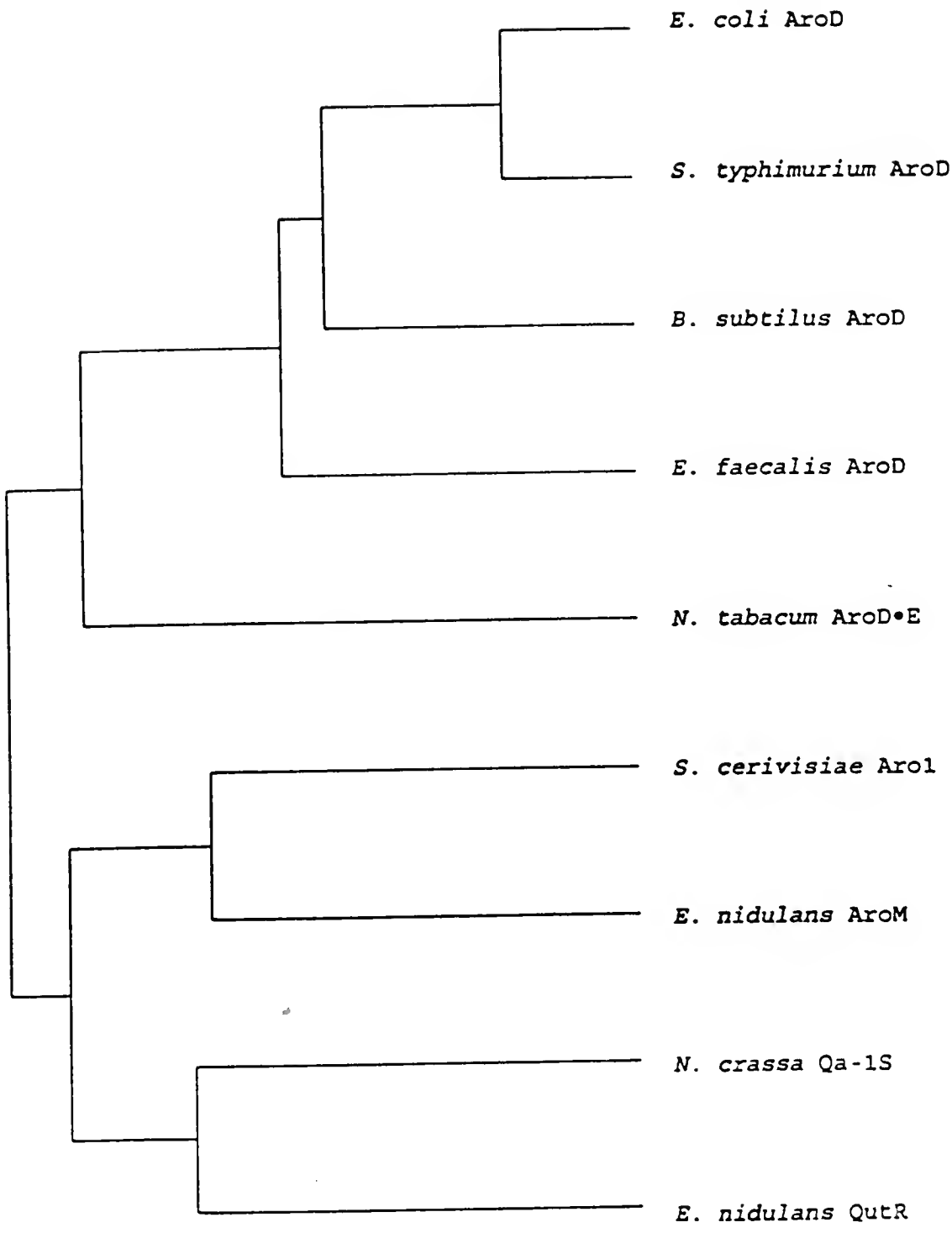

E. coli AroD
S. typhimurium AroD
B. subtilus AroD
E. faecalis AroD
N. tabacum AroD•E
S. cerivisiae Aro1
E. nidulans AroM
N. crassa Qa-1S
E. nidulans QutR

Fig. 7-7. The latter shows two clusters, with Nta-AroD•E grouping with the monofunctional proteins of the prokaryotes: *E. coli, Salmonella typhimurium, Bacillus subtilis,* and *Enterococcus faecalis.* Thus, the bifunctional plant enzyme is evolutionarily closer to the prokaryote monofunctional proteins than to the AroD domains of the pentafunctional proteins present in the eukaryotes, yeast (Aro1) and fungi (AroM).

The proteins in the multiple alignment shown in Fig. 7-6 are separated between the two clusters that are shown in Fig. 7-7 for clarity. This alignment shows the conserved residues within each cluster, as well as those conserved throughout both clusters. Three amino acids shown to be important catalytic residues for the *E. coli* AroD protein, HIS (25), MET (47), and LYS (20) are marked with asterisks, and these are conserved in all proteins having AroD catalytic activity. It has been suggested that the repressor proteins from *Neurospora crassa* (Qa-1S) and from *Emericella nidulans* (QutR) evolved from three of the five domains of the pentafunctional protein corresponding to dehydroquinase, shikimate dehydrogenase, and shikimate kinase (38). These retained the ability to bind what previously were substrate molecules to function in a new role as regulatory agents. Thus, critical residues such as those marked by asterisks were altered to retain binding but to lose catalysis. Note that nine other residue positions

are completely conserved in the catalytic proteins, but not in the two regulatory proteins. This implies that some or all of these residues may be important for catalysis. Six residues are absolutely conserved for all nine proteins.

Homology relationships of the SDH domain

A multiple alignment is shown in Fig. 7-8 of the AroE domain of the S-protein with its homologues. The corresponding dendrogram is shown in Fig. 7-9. Again two evolutionary clusters are seen in the dendrogram, and the AroE domain clusters with the monofunctional AroE protein of *E. coli*, rather than with the AroE domains of the pentafunctional proteins (Aro1 and AroM) or with the repressor proteins (Qa-1S and QutR). The *Nicotiana* AroD domain is also homologous with the catabolic quinate dehydrogenase of yeast and fungi, which are monofunctional and specific for NAD^+. However, the latter (QutB and Qa-3) are evolutionarily closer to the repressor proteins (Qa-1S and QutR) than to the shikimate dehydrogenase domain of the plant bifunctional S-protein. It is surprising that the catabolic QDH exhibits homology with biosynthetic SDH while the catabolic dehydroquinases display no obvious homology with biosynthetic dehydroquinases (32, 35). This is all the more striking in that the former differ in substrate specificity, whereas the latter do not. One might also have expected the catabolic dehydroshikimate dehydratase to be homologous with the biosynthetic dehydroquinases or

Fig. 7-8. Multiple amino acid alignment of the shikimate dehydrogenase domain of AroD•E and its homologues. Nine proteins have been aligned and conserved regions are boxed. The probable domain for the NADPH binding site is underlined. The amino acid residue numbers for each sequence are on the right of the figure:

Designation	Organism	Protein	Accession Number
Eni-QutB	*Emericella nidulans*	quinate dehydrogenase	P25415
Ncr-Qa-3	*Neurospora crassa*	quinate dehydrogenase	P11635
Ncr-Qa-1S	*Neurospora crassa*	quinate repressor protein	P11637
Eni-QutR	*Emericella nidulans*	quinate repressor protein	M59935
Sce-Aro1	*Saccharomyces cerivisiae*	arom pentafunctional protein	P08566
Eni-AroM	*Emericella nidulans*	arom pentafunctional protein	P07547
Eco-AroE	*Escherichia coli*	dehydroquinase	P15770
Nta-AroD•E	*Nicotiana tabacum*	bifunctional S-protein	-
Eco-URF	*Escherichia coli*	unidentified reading frame	P28244

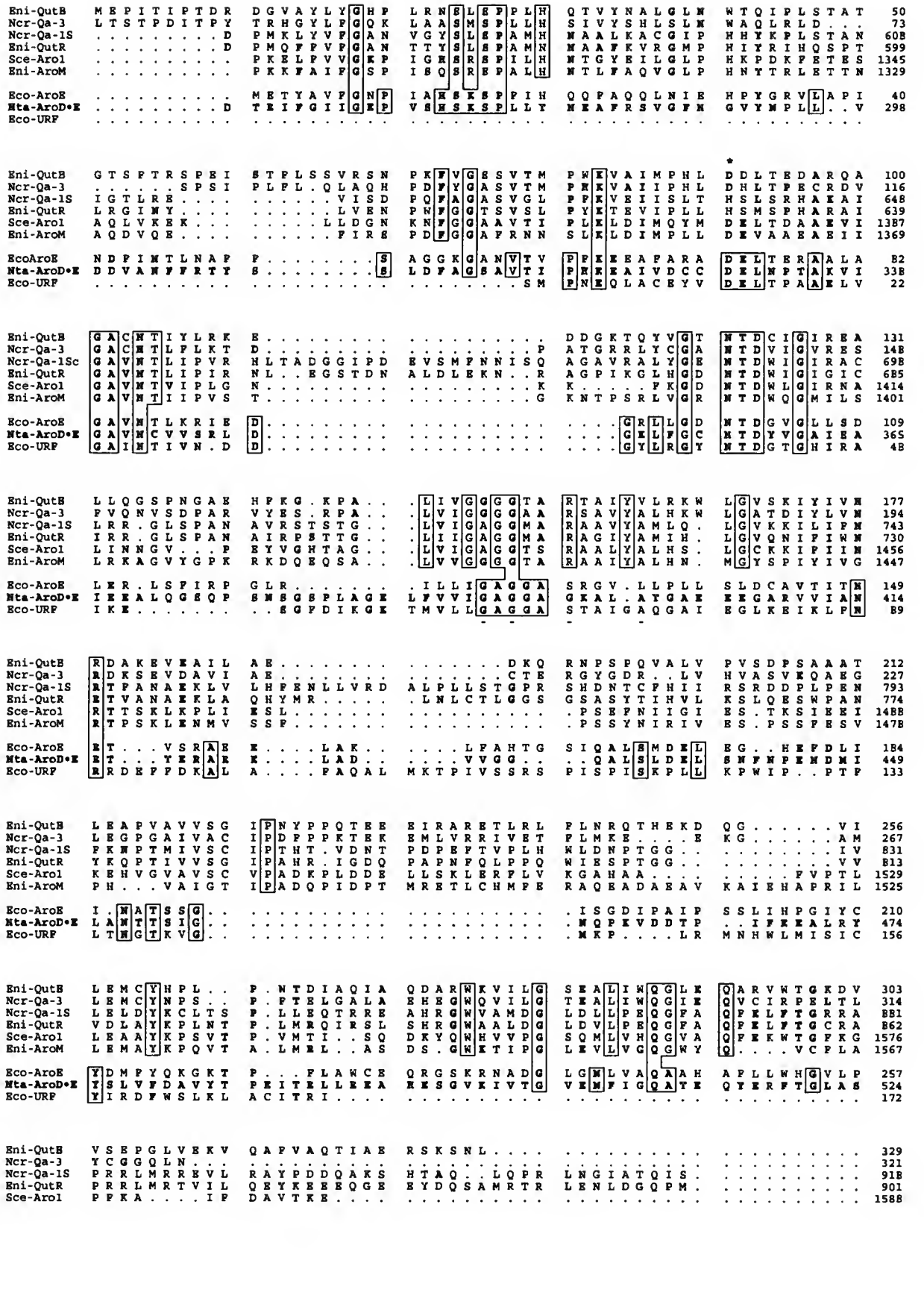
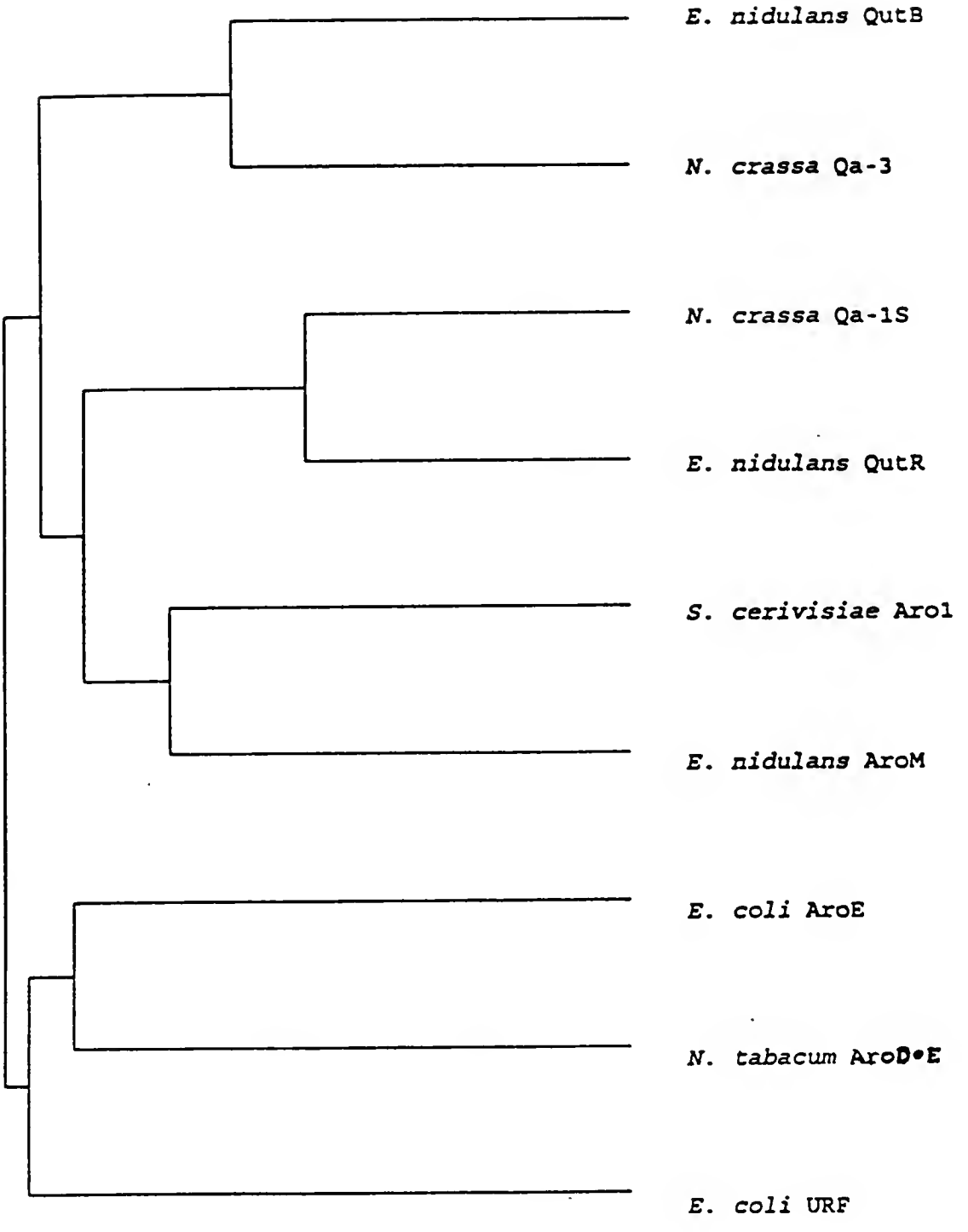


Fig. 7-9. Dendrogram of shikimate dehyrogenase homologous. The dendrogram is based on the multiple amino acid alignment and includes the nine proteins defined in the legend of Fig.7-8.


E. nidulans QutB
N. crassa Qa-3
N. crassa Qa-1S
E. nidulans QutR
S. cerivisiae Aro1
E. nidulans AroM
E. coli AroE
N. tabacum AroD•E
E. coli URF

catabolic dehydroquinases. Table 7-2 shows each pairwise comparison of *N. tabacum* AroD•E with its homologues (Gap program).

Table 7-2. Pairwise comparisons of S-protein with its homologues.

Organism and Gene	Percent			
	DQT		SDH	
	Identity	Similarity	Identity	Similarity
Sce-Aro1	29	53	28	56
Eni-AroM	24	52	27	51
Eco-AroD	30	52	-	-
Eco-AroE	-	-	31	55
Sty-AroD	29	51	-	-
Efa-AroD	34	54	-	-
Bsu-AroD	32	53	-	-
Ncr-Qa-1S	22	44	30	53
Eni-QutR	21	46	26	47
Eni-QutB	-	-	28	53
Ncr-Qa-3	-	-	26	50
Eco-URF	-	-	36	61
Stu-MDH	-	-	23	51
Nta-CDH	-	-	21	47

NAD(P)H-binding proteins often share a motif (Rossman fold) dominated by glycine or other residues with small sidechains such as alanine (72). Table 7-3 shows a comparison of this region in the organisms compared in this dissertation with a number of others recovered by the GCG Blast program. The S-protein fits the NADPH binding motif perfectly with GxGxxAxxxA (72).

A survey of some chloroplast transit peptides

Table 7-3. NAD(P)H Binding Motif of Enzymes[a].

Organism	cDNA or Gene Product	Conserved Region GxGxxGG GxGxxVxxxA GxGxxAxxxA	Accession Number
E. nidulans	Quinate repressor (QutR)	STTGLIIGAGGMARAGIYAM	M59935
N. crassa	Quinate repressor (Qa-1S)	TSTGLVIGAGGMARAAVYAM	P11637
E. nidulans	Quinate dehydrogenase (QutB)	GKPALIVGGGGTARTAIYVL	P25415
N. crassa	Quinate dehydrogenase (Qa-3)	SRPALVIGGGGAARSAVYAL	P11635
E. nidulans	Shikimate dehydrogenase (AroM)	EQSALVVGGGGTARAALYAL	P07547
S. cerevisiae	Shikimate dehydrogenase (Aro1)	HTAGLVIGAGGTSRAALYAL	P08566
E. coli	Shikimate dehydrogenase (AroE)	GLRILLIGAGGASRGVLLPL	P15770
E. coli	unidentified reading frame	GKTMVLLGAGGASTAIGAQG	P28244
N. tabacum	S-protein (AroD•E)	GKLFVVIGAGGAGKALAYGA	-
S. tuberosum	Malate dehydrogenase	KMKIVVAGAGSAGIGVLNAA	Z23023
N. tabacum	Cinnamyl-Alcohol dehydrogenase1	GFRGGILGLGGVGHMGVKIA	P30360
N. tabacum	Cinnamyl-Alcohol dehydrogenase2	GLRGGILGLGGVGHMGVKIA	P30359
H. vulgare	Alcohol dehydrogenase1	GSTVAIFGLGAVGLAAAEGA	P05336
T. repens	Alcohol dehydrogenase1	GSSVAIFGLGAVGLAAAEGA	P13603
C. roseus	S-Adenosylhomocysteinase	GKVAVVAGYGDVGKGCAAAL	P35007
T. aestivum	S-Adenosylhomocysteinase	GKVAVVCGYGDVGKGCAAAL	P32112
N. silvestris	S-Adenosylhomocysteinase	GKVALVAGYGDVGKGCAAAL	D16138
H. sapiens	Alcohol dehydrogenase gamma	GSTCAVFGLGGVGLSVVMGC	P00326
H. sapiens	Glutathione reductase	SYDYLVIGGGSGGLASARRA	S08979
M. musculus	Lactate dehydrogenase	NNKITVVGVGQVGMACAISI	P16125
P. stipitis	*D*-Xylulose reductase	GDYVAVFGAGPVGLLAAAVA	P22144
S. cerevisiae	Succinate dehydrogenase	VVIGAGGAGLRAAFGL	Q00711
K. marxianus	Alcohol dehydrogenase1	TKLPLVGGHEGAGVVVAMGE	P20369
C. synechocystis	Ketolate reductoisomerase	GKTVAIIGYGSQGHAHALNL	A47037
E. coli	Threonine dehydrogenase	GEDVLVSGAGPIGIMAAAVA	P07913
E. coli	Glutamate synthase	GKKVAIIGAGPAGLACADVL	B29617
T. brucei	Phosphogluconate DH	VVGLGVMGANLALNI	P31072
T. brockii	NADH oxidase	KKVVVVGGGPAGMQAAITA	P32382
P. putida	Formaldehyde DH	GSTVYVAGAGPVGLAAAASA	D21201

[a]Motifs were taken from Scrutton et al., 1990. The first nine set of organisms are those used in the SDH multiple allignment of Fig. 7-8.

established in the literature is shown in Table 7-4. The transit peptides vary from 34 to 88 amino acid residues. The sequence context around the translation start (AUG) is proposed to most often be AACAAUGGC in plant genes (54). Included in this survey are the seven known sequences for aromatic pathway proteins. From this selection in Table 7-4, only one of sixteen, chorismate synthase in barley (aromatic pathway protein) has this proposed conserved region. The cleavage site often exhibits the pattern (V/I)-X-(A/C)↓A, with R frequently at position -2 or between -6 to -10 (33). Of the seven aromatic pathway proteins, shikimate kinase in tomato conforms to the motif before the cleavage point and five of the remaining nine sequences conform to the cleavage point (two of these also have the proposed ALA after the cleavage point). The S-protein does not fit this cleavage site motif. The overall features of transit peptides, show that they are rich in hydroxylated amino acids, SER and THR (20-35%), small hydrophobic amino acids such as VAL and ALA, not especially rich in basic amino acids (but do have a net positive charge), and usually have only one or two acidic groups (45). The aromatic pathway protein transit peptides follow these features, being especially rich in SER. The S-protein truncated transit peptide (23 residues) has 17% SER (3) and THR (1), 35% hydrophobic residues, 0.09% basic residues {R (ARG) and K (LYS) near the cleavage point} and 13% acidic residues.

Table 7-4. Transit Peptides of Aromatic Pathway proteins and of other Plant Proteins.

Enzyme Plant	Start Transit Peptide---------->	Amino Acid Number	Mature Protein
DAHPsyn agcaatggc potato	MALSSTSTTNSLLPNRSLVQNQPLLPSPLKNAFFSNNSTKTVRFVQPISAVHSSDSNKIPIVSDKPSKSSPPAA	74	TATTAP..
EPSPsyn agaaatggc tomato	MAQISSMAQGIQTLSLNSSNLSKTQKGPLVSNSLFFGSKKLTQISAKSLGVFKKDSVLRVVRKSSFRISASVATAE	76	KPHEIV..
EPSPsyn aagaatggc petunia	MAQINNMAQGIQTLNPNSNFHKPQVPKSSSFLVFGSKKLLNSANSMLVLKKDSIFMQKFCSFRISASVATAQ	72	KPSEIV..
EPSPsyn tccgatggc cruc. weed	MAQVSRICNGVQNPSLISNKSSQRKSPLSVSLKTQQHPRAYPISSSWGLKKSGMTLIGSELRPLKVMSSVSTAE	74	KASEIV..
CHAsyn aacaatggc barley	MASSLSTKPPLSGSRRRSTTDGSGWSYFQTSDLRQLSNQSVQISVRRQTAPLKLVVQASG	57	ASGSSF..
SHKkin gactatgga tomato	MEARVSQSLQLSSWINSDKVVRKPSGLLRFSEKWNEKPRHRVVVSCHLQPRKAAHSDRRVQLKVSC	65	SPQNVQ..
TRPsyn agtaatggc cruc. weed	MAASGTSATFRASVSSAPSSSSQLTHLKSPFKAVKYTPLPSSRSKSSSFSVSCTIAKDPPVLMAAGSDPALW	72	QRPDSF..
ASPat aaacatggc soybean	MASSFLSAASHAVSPSCSLSTTHKGKPMLGGNTLRFHKGPWSFSSSRSRGRISMAVAVNV	60	SRFEGI..
GSAat catcatggc barley	MAGAAAAVASGISIRPVAAPKISRAPRSRSVVRA	34	AVSIDE..
L12ribp tacaatggc tobacco	MASTLSTITLRSPSPSTASSTHASIPFPKKALEFPIRTPKLHHRRAT	53	FLRPLA..
IPMdh tgaaatggc rape	MAAALQTNIRPVKFPATLRALTKQSSPAPFRVRC	33	AAASPG..
PCPoxre agtaatggc pea	MALQTASMLPASFSIPKEGKIGASLKDSTLFGVSSLSDSLKGDFTSSALRCKRELRQKVGAVRA	64	ETAAPA..
FEDred cgccatggc spinach	MTTAVTAAVSFPSTKTTSLSARSSSVISPALISYKKVPLYYRNVSATGKMGPIRA	55	QIASDV..
FBPald taagatggc spinach	MASASLLKTSPVLDNPGFLKGQTLRIPSVAGVRFTPSGSSSLTVRA	46	SSYADG..
CARanhy accaatgtc pea	MSTSSINGFSLSSLSPAKTSTKRTTLRPFVFASLNTSSSSSSSSTFPSLIQDKPVFASSS	60	PIITPV..
G3Pacyt gggatgac pea	MTDSFAHCASHINYRHKMKTMPIFSTPCCSPSTAFFSPFRASNSKPLRSTLSLRSSISSSSITSTSHCSLAFNI IVKHKEKNVVSANMT	88	SSVSSR..
S-PROT not known tobacco	PFLCSTSWLLMELVVDSGVRKME	XX	GEAMRR..

SUMMARY

In conclusion, with the cloning of the *aroD•aroE* cDNA encoding the S-protein (the first to be cloned and sequenced in higher plants), other studies may now be undertaken. First of all, a complete sequence is desirable, since it is proposed that the 5′ region has been truncated. This may be accomplished by obtaining mRNA from *Nicotiana tabacum* plants for primer extension. Other studies may include cloning of the gene from a genomic library, determining the number of gene copies in *Silvestris* or in other plant species by use of Southern blots, performing site directed mutagenesis or chemical treatment to study the active site residues, use of the cloned cDNA coding for the S-protein as a probe to obtain the cDNA from other plants and to determine what the cytosolic pathway has with respect to dehydroquinase and shikimate dehydrogenase. The probable location of the bifunctional gene product in the chloroplast remains to be proven. Clarification of the relationship of SP-I and SP-II is needed. The possibilities are: (i) that one is an artifact of limited proteolysis, (ii) that one is an active, uncleaved preprotein in the cytosol, (iii) or that they are separately subcompartmented in the chloroplast.

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I certify that I have read this study and in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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